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ANA CARLA ZARPELON

PARTICIPAÇÃO DA IL-33 EM MODELOS DE RESPOSTA INFLAMATÓRIA INATA E DOR NEUROPÁTICA EM CAMUNDONGOS

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Orientador: Prof. Dr. Waldiceu Aparecido Verri Jr.

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“Se não puder voar, corra.
Se não puder correr, ande.
Se não puder andar, rasteje,
mas continue em frente de qualquer jeito.”

Martin Luther King

Resumo

ZARPELON, A.C. Participação da IL-33 em modelos de resposta inflamatória inata e dor neuropática em camundongos. Tese – Departamento de Patologia, Centro de Ciências Biológicas – Universidade Estadual de Londrina.

A IL-33 é um membro da família de citocinas IL-1, que sinaliza via receptor ST2. Essa citocina está envolvida na dor induzida pela resposta imune adaptativa, entretanto, pouco se sabe a respeito do papel da IL-33 em modelos de resposta imune inata e dor neuropática. Nesse trabalho demonstramos os mecanismos pelos quais a IL-33 contribui para inflamação inata induzida pela carragenina. Além disso, foi investigado o papel da IL-33 em modelo de neuropatia induzido pela injúria por constrição crônica do nervo ciático em camundongos. No primeiro momento, avaliamos se a carragenina e a IL-33 eram capazes de induzir a resposta inflamatória em animais BALB/c (selvagem) e deficientes para o receptor ST2 através da avaliação dos parâmetros: edema, atividade de mieloperoxidade, hiperalgesia mecânica, citocinas, expressão de RNA mensageiro e tratamentos farmacológicos inibindo o recrutamento leucocitário (fucoidina), TNFα (infliximab), CXCL1 (anticorpo contra CXCL1), IL-1 (antagonista do receptor da IL-1), endotelinas, receptor ET_A (clazosentan) e ET_B (BQ788) e ciclooxigenase (indometacina). A fim de avaliar o papel da IL-33 na dor neuropática, a hiperalgesia mecânica também foi avaliada, os níveis de citocinas foram determinados por ELISA, ativação de proteínas foi determinada pelo método de western blot e, além disso, foram utilizadas ferramentas farmacológicas para alvos específicos. A injúria por constrição crônica foi induzida por ligação no nervo ciático. Diferenças estatísticas foram consideradas significativas para $p<0.05$. Os procedimentos foram aprovados pelo Comitê de Ética da Universidade de São Paulo e Universidade Estadual de Londrina. Os resultados demonstraram que a administração de carragenina aumentou a expressão de RNAm para ST2 e IL-33 e a produção de IL-33 em amostras de pele de pata. Além disso, a administração de carragenina na pata induziu edema, hiperalgesia mecânica e atividade de mieloperoxidase, que foi reduzida nos animais $ST2^{-/-}$ em comparação com os selvagens, os mesmos efeitos foram observados pela administração de IL-33 na pata. A hiperalgesia induzida pela

IL-33 foi reduzida pelo tratamento com fucoidina, sugerindo o papel do recrutamento de leucócitos no efeito hiperalgésico. Em animais naïve a hiperalgesia induzida pela IL-33 foi reduzida pelos tratamentos farmacológicos para os seguintes alvos, TNF-α, CXCL1, IL-1, receptores de endotelina e ciclooxigenase, enquanto que a produção de TNF-α, CXCL1, IL-1β, IL-10 e PGE_2, bem como, a expressão de RNAm para prepro-ET-1 induzidos pela carragenina foram dependentes do receptor ST2. A administração combinada de IL-33 e carragenina, em doses submáximas em tratamento único, induziu hiperalgesia, edema, atividade de mieloperoxidase e produção de citocinas de maneira dependente de ST2. Investigando o papel da IL-33 na dor neuropática, foi demonstrado que a injúria por constrição crônica induz uma produção significativa de IL-33 na medula espinal (L4-L6) e aumento da hiperalgesia durante 20 dias, começando 3 dias após a cirurgia, a qual foi inibida em camundongos deficientes para ST2. A administração intratecal (i.t.) de IL-33 induziu a hiperalgesia de maneira dose-dependente, que foi inibida nos camundongos $ST2^{-/-}$ e $TNFR1^{-/-}$ e pelo tratamento com o antagonista do receptor da IL-1, bem como induziu a produção espinal de TNFα and IL-1β. A hiperalgesia induzida pela administração i.t. de IL-33 foi reduzida pelo tratamento com inibidores de PI3K, mTOR, MAP kinases (p38, ERK e JNK) e NFκB. Corroborando, a injúria por constrição crônica induz a ativação espinal de PI3K, AKT, mTOR, MAP kinases e NFκB, verificado pelo método de western blot, o que foi reduzido nos camundongos $ST2^{-/-}$. O tratamento com minociclina e fluorocitrato inibiu a hiperalgesia induzida pela IL-33 e pela injúria por constrição crônica, e nos animais deficientes para o $ST2^{-/-}$, houve redução na ativação de GFAP e Iba-1 na medula espinal. Dessa forma, a sinalização IL-33/ST2 é importante na produção de mediadores inflamatórios contribuindo para a inflamação induzida pela carragenina. Além disso, a via IL-33/ST2 medeia a dor induzida pela injúria por constrição crônica pela ativação de mecanismos espinais importantes, que incluem PI3K/AKT, mTORC1 e 2, MAPK (p38, ERK e JNK) e NFκB. Esses dados reforçam a importância da sinalização IL-33/ST2 como alvo na dor inflamatória e neuropática.

Palavras-chaves: IL-33, carragenina, dor neuropática, sinalização espinal e células da glia.

Abstract

ZARPELON A.C., Role of interleukin-33 in models of innate immune inflammation and neuropathic pain in mice. Thesis – Department of Pathology, Biological Sciences Center – Universidade Estadual de Londrina.

IL-33 is a member of IL-1 family that signals through ST2 receptor. This cytokine is involved in adaptive inflammation-induced pain, however, the role of IL-33 on innate immune inflammation and neuropathic pain remains unknown. We report here the contribution of IL-33/ST2-triggered mechanisms to carrageenin-induced innate inflammation. Furthermore, it was investigated the role of IL-33 in chronic constriction injury of the sciatic nerve (CCI)-induced neuropathic pain in mice. In a first set of experiments, carrageenin- and IL–33-induced inflammatory responses were assessed in BALB/c- (WT) and ST2-deficient ($^{-/-}$) mice as follows: oedema, myeloperoxidase activity, mechanical hyperalgesia, cytokine levels, PGE_2, mRNA expression, drug treatment targeting leukocyte recruitment (fucoidin), TNF-α (infliximab), CXCL1 (antibody to CXCL1), IL-1 (IL-1ra), endothelin ET_A (clazosentan) and ET_B (BQ788) receptors and COX (indomethacin). In orther to evaluate the role of IL-33 in neuropathic pain, mechanical hyperalgesia was also evaluated. Moreover, cytokine levels were determined by ELISA, protein activation was determined by western blot and specific targets were inhibited by pharmacological tools. CCI was induced by one ligation of the sciatic nerve. Statistical differences were considered significant for $P<0.05$. Procedures were approved by the Ethics Committee of University of Sao Paulo and Londrina State University. The result of our first study demonstrated that carrageenin injection increased ST2 and IL-33 mRNA expression and IL-33 production in paw skin samples. Furthermore, carrageenin-induced paw oedema, hyperalgesia and myeloperoxidase activity were reduced in ST2$^{-/-}$ compared with WT mice as well as carrageenin effects were mimicked by IL-33 injection in the paw. Furthermore, IL–33-induced hyperalgesia was reduced by fucoidin suggesting a role for recruited leukocytes in its hyperalgesic effect. IL–33-induced hyperalgesia in naïve mice was reduced by treatments targeting TNF, CXCL1, IL-1, endothelin receptors and cyclooxigenase while carrageenin-induced

ST2-dependent TNFα, CXCL1, IL-1β, IL-10 and PGE2 production and preproET-1 mRNA expression. Combining IL-33 and carrageenin at doses that were ineffective as single treatment induced significant hyperalgesia, oedema, myeloperoxidase activity and cytokine production in a ST2-dependent manner. Investigating the role of IL-33 in neuropathic pain, we demonstrate that CCI induced significant IL-33 production in the spinal cord (L4-L6) and hyperalgesia during 20 days starting 3 days after surgery, which was inhibited in ST2$^{-/-}$ mice. intrathecal (i.t.) injection of IL-33 induced dose-dependent hyperalgesia, which was inhibited in ST2$^{-/-}$ and TNFR1$^{-/-}$ mice and by IL-1ra as well as IL-33 induced spinal production of TNFα and IL-1β. The hyperalgesia induced by i.t. injection of IL-33 was diminished by inhibitors of PI3K, mTOR, MAP kinases (p38, ERK and JNK) and NFκB. In agreement, CCI-induced spinal activation of PI3K, AKT, mTOR, MAP kinases and NFκB were reduced in ST2$^{-/-}$ mice compared to WT mice as determined by western blot. The treatment with minocicline and fluorocitrate inhibited IL-33-induced hyperalgesia and CCI-induced spinal activation of GFAP and Iba-1 were inhibited in ST2$^{-/-}$ compared to WT mice. Therefore, IL-33/ ST2 signaling triggers the production of inflammatory mediators contributing to carrageenin-induced inflammation. Furthermore, IL-33/ST2 signaling pathway mediates chronic constriction injury-induced pain by activating important spinal mechanisms including PI3K/AKT, mTORC1 and 2, MAPK (p38, ERK e JNK) and NFκB. These data reinforces the importance of IL-33/ST2 signalling as a target in inflammatory and neuropathic pain.

Key words: IL-33, carrageenin, neuropathic pain, spinal signaling and glial cells.

SUMÁRIO

1. Introdução

1.1. Dor Inflamatória

A injúria tecidual resulta em inflamação e dor inflamatória, tais como as dores articulares, dor pós-operatória e injúria do nervo ciático (JI et al., 2011). A dor é uma experiência complexa que envolve não somente a transdução de sinais prejudiciais no microambiente, mas também alterações cognitivas e emocionais (JULIUS & BASBAUM et al., 2001). Atualmente a dor é definida, pela Associação Internacional para o Estudo da Dor (IASP), como sendo “uma experiência emocional e sensorial desagradável associada ou não com lesão tecidual real, ou potencial, ou descrita em termos de tal lesão”.

A capacidade do organismo em reconhecer a existência de um processo danoso torna a dor um sintoma clinicamente importante, pois proporciona um “sinal de alerta”. Entretanto, de forma inapropriada através do reconhecimento de componentes do organismo como sendo não próprios pelo sistema imune, a inflamação acaba sendo prejudicial, contribuindo para o estabelecimento das lesões teciduais.

A dor inflamatória resulta da sensibilização e ativação dos neurônios periféricos por mediadores inflamatórios finais, tais como as prostaglandinas e aminas simpáticas. Os produtos das ciclooxigenases (COX), as prostaglandinas, principalmente da série E, são metabólitos resultantes da ação das COXs sobre o ácido araquidônico. Em condições fisiológicas, o ácido araquidônico encontra-se esterificado nos fosfolipídeos de membrana, e durante o processo inflamatório a enzima fosfolipase A_2 é capaz de mobilizá-lo, sob a ação de estímulos químicos, mecânicos e produtos microbianos (FERREIRA & VANE, 1967). O reconhecimento desses mediadores se dá por diferentes receptores presentes no neurônio aferente primário ou nociceptor. Além das prostaglandinas (PGE_2), que são capazes de induzir dor (FERREIRA et al., 1972), as aminas simpáticas (noradrenalina, adrenalina e dopamina) são capazes de induzir nocicepção em ratos (NAKAMURA E FERREIRA, 1987). A liberação dos mediadores finais, geralmente é precedida pela liberação dos mediadores intermediários, sendo os principais, as citocinas, como o fator de necrose tumoral (TNF-α), interleucina (IL)-1β e IL-8.

O termo nociceptor, pode ser utilizado para definir o neurônio primário como um todo, ou somente suas terminações nervosas livres, sendo que neste trabalho, quando se utilizar esse termo, estará tratando do neurônio nociceptivo (RAHMAN et al, 2013). A estimulação dos nociceptores faz com que a informação nociceptiva seja conduzida através das fibras aferentes primárias (neurônios de primeira ordem) ao sistema nervoso central (SNC), as quais estão subdividas em três principais grupos. As primeiras possuem amplo diâmetro de corpo celular, são altamente mielinizadas e conduzem o estímulo rapidamente, sendo conhecidas como fibras Aβ. Estas fibras são capazes de detectar estímulos inócuos aplicados à pele e músculos, entretanto não contribuem para dor. Por outro lado, existem as fibras de baixo e médio diâmetro de corpo celular, sendo que as fibras C não são mielinizadas e conduzem o estímulo lentamente, e as finamente mielinizadas que conduzem os estímulos mais rapidamente, chamadas de fibras Aδ. Desde então, tem se assumido que as fibras Aδ e C, medeiam a primeira e segunda dor, respectivamente. Além disso, a atividade de algumas fibras C é devidamente difícil de identificar, e são chamadas de “silenciosos” ou “dormentes”, respondendo somente quando são sensibilizados pela injúria do tecido (JULIUS & BASBAUM et al., 2001).

Os mediadores inflamatórios causam a sensibilização dos neurônios aferentes primários e consequente diminuição do limiar de ativação desses. Esse aumento de excitabilidade dos neurônios aferentes contribui para hipersensibilidade a dor, a qual é caracterizada por três fenômenos principais: hiperalgesia (resposta exacerbada a um estímulo doloroso), alodinea (resposta nociceptiva a estímulos anteriormente não dolorosos) e dor espontânea.

Os terminais periféricos ao nociceptor expressam receptores para os diferentes mediadores inflamatórios, e a ativação destes causa à hiperatividade de moléculas de transição, tais como o receptor de potencial transitório subtipo V1 (TRPV1) e A1 (TRPA1), e moléculas de condução tais como os canais de sódio Nav 1.7, 1.8 e 1.9. Como resultado a sensibilidade e excitabilidade dos nociceptores é aumentada, via ativação de proteínas quinases, tais como proteína quinase A (PKA), PKC e proteínas quinases ativadas por mitógenos (MAPK) (JI et al., 2011; BASBAUM et al., 2009).

Até o momento foram identificados nove subtipos de canais de sódio, os Nav 1.1, 1.6 – 1.9 são altamente expressos no gânglio da raiz dorsal (DRG), sendo que os canais Nav 1.7 – 1.9 estão presentes preferencialmente nos neurônios periféricos, portanto extremamente associados às condições de dor inflamatória crônica. Além dos canais de sódio, os canais de cálcio também têm grande potencial como mediadores da dor e inflamação nos nociceptores periféricos. Os canais de sódio e cálcio estão sendo estudados a fim de estabelecer bloqueadores seletivos, objetivando estratégia terapêutica para o tratamento das dores crônicas (RAHMAN et al., 2013).

As respostas desordenadas decorrentes da diminuição do limiar de excitabilidade neuronal e consequente ativação de proteínas quinases contribuem para a evolução da inflamação a qual pode envolver as condições presentes na dor neuropática, além disso, a persistência e severidade progridem de acordo com o dano neuronal e extensão do dano tecidual (RAHMAN et al., 2013).

Além dos mediadores inflamatórios que induzem e propulsionam a dor inflamatória, vários estudos tem relacionado a presença dos neutrófilos em doenças inflamatórias, evento que é prejudicial ao organismo, contribuindo para o estabelecimento das lesões tissulares (WEISSMANN & KORCHAK, 1984; WEISS, 1989). O processo da migração de neutrófilos durante a resposta inflamatória é intermediado por diferentes mediadores inflamatórios e quimiotáticos, os quais promovem aumento nas interações de neutrófilos com células endoteliais, favorecendo a migração dos leucócitos das vênulas pós-capilares para o tecido inflamado (HUTTENLOCHER et al., 1995).

Uma vez presente no sítio inflamatório, os neutrófilos são capazes de fagocitar, destruir e degradar os microrganismos, sendo a destruição destes realizada por mecanismos dependentes de oxigênio, nitrogênio e enzimas proteolíticas. Entretanto, as quantidades de peróxido de hidrogênio formado, não são capazes de induzir destruição eficaz dos microrganismos. No entanto, os grânulos azurófilos (lisossomos verdadeiros ou grânulos primários) dos neutrófilos contêm a enzima mieloperoxidase (MPO), que na presença de íons Cl^-, converte o peróxido de hidrogênio em hipoclorito, sendo este importante para a destruição do agente injuriante (KLEBANOFF et al., 1970).

1.2. Resposta inflamatória inata

Dentre os modelos utilizados para resposta inflamatória inata, destaca-se a administração intraplantar de carragenina. A carragenina é um polissacarídeo sulfatado com massa molecular relativa acima de 100 KDa, derivado de algas marinhas vermelhas da classe Rhodophyceae. A carragenina não tem valor nutritivo, contudo tem sido largamente utilizada na presença de alimentos durante séculos. Ela apresenta propriedades de gelificação, espessamento, emulsificantes e solubilização de alimentos processados. Em 1969, foi concedido pelo FDA (Food and drugs administration) um certificado de substância segura para ser incorporada em produtos alimentares, desde que utilizada em quantidades suficientes apenas para obter o efeito desejado.

A carragenina é muito utilizada em modelos experimentais de doenças inflamatórias devido a sua intensa capacidade de induzir inflamação local. Diversos grupos tem sugerido que o uso crônico da carragenina pode estar associado diretamente à gênese das neoplasias e ulcerações intestinais. No entanto, ela continua sendo empregada como aditivo alimentar (MARCUS & WATT, 1981; MARCUS, 1981; EKSTROM et al., 1983).

Sabe-se que a imunidade inata consiste em barreiras que impedem a entrada de materiais nocivos ao organismo, dentre elas a pele e o muco, entretanto, se um antígeno ultrapassar as barreiras externas entram em ação outros mediadores do sistema imune inato, tais como as proteínas do complemento e a família dos interferons. A resposta inflamatória faz parte da imunidade inata, pois o tecido lesado é capaz de liberar mediadores químicos como a histamina, bradicinina e serotonina a fim de eliminar o agente agressor.

A hiperalgesia inflamatória é o resultado de modificações funcionais nos neurônios aferentes primários, por ativação metabotrópica que se extende por todo neurônio sensitivo (sensorial). Essas modificações funcionais da excitabilidade neuronal são induzidos por mediadores liberados pelo trauma tecidual ou pelo reconhecimento de produtos resultantes do trauma por células do sistema imunológico, como os neutrófilos e macrófagos. Dessa forma, compreende-se que a liberação de mediadores inflamatórios e a migração de células para o local da injúria são os responsáveis pelos sinais e sintomas inflamatórios (CUNHA et al., 2005; VERRI et al., 2006).

Diferentes tipos celulares, tais como células dendríticas (DCs), macrófagos, linfócitos e células da glia, são capazes de reconhecer um estímulo inflamatório, desencadeando a liberação de uma cascata de citocinas essenciais para o desenvolvimento da inflamação e dor inflamatória, tais como IL-1β, TNF-α, IL-6, citocina indutora da quimioatração de neutrófilos (CINC-1) e quimiocina derivada de queratinócitos (KC), que podem desencadear a produção de outros mediadores.

Atualmente descreve-se que as citocinas são mediadores que permitem uma ligação entre a injuria celular, reconhecimento imunológico e a presença de sinais sistêmicos e locais da inflamação (CUNHA & FERREIRA, 2003; CONTI et al., 2004). A maioria da citocinas apresenta funções múltiplas e são capazes de responder a uma diversidade de estímulos (VERRI et al., 2006). No modelo da carragenina, Cunha e colaboradores em 2005 descreveram a presença de mediadores intermediários (citocinas e quimiocinas) e finais (prostaglandinas e aminas simpáticas), os quais são capazes ou não de sensibilizar diretamente os nociceptores.

Nesse contexto, nosso grupo demonstrou que durante o processo inflamatório, induzido pela carragenina, a hipernocicepção mecânica é precedida pela liberação de citocinas, principalmente pelo TNF-α e CXCL1/KC, as quais são capazes de induzir IL-1β, que por sua vez, é responsável pela estimulação da síntese de prostaglandinas e consequente liberação de aminas simpáticas (CUNHA et al., 2005). Evidências sugerem que os polissacarídeos derivados de algas marinhas regulam a imunidade inata através da ligação de receptores de reconhecimento de padrões (PRRs), tais como os receptores de manose (receptores do tipo toll - (TLRs)) (TSUJI et al., 2003).

Existem diversas classes de receptores presentes na membrana dos neutrófilos, incluindo receptores de sete α-hélices acoplados a proteína G, receptores Fc, receptores para uma série de citocinas, e os receptores da resposta imune inata, tais como TLRs e lectinas. A ligação aos receptores leva a ativação de um complexo celular culminando em fagocitose, liberação de grânulos intracelulares, produção de espécies reativas de oxigênio, liberação de armadilhas extracelulares de neutrófilos (NETs), migração quimiotática e produção de citocinas e quimiocinas (FUTOSI et al., 2013).

Na investigação de uma nova citocina, é importante utilizar um modelo amplamente conhecido a fim de avaliar mecanismos já estudados nesse modelo, dessa forma, utilizamos o modelo da carragenina para investigação da participação da IL-33 na inflamação inata. Nosso grupo já desempenhou outros trabalhos com o mesmo estímulo, evidenciando a atividade anti-inflamatória de algumas drogas, tais como a budleína A (VALERIO et al., 2007), frutose-1,6-bifosfato (VALERIO et al., 2009), quercetina (VALERIO et al., 2009b), ácido caurenóico (MIZOKAMI et al., 2012), sal de Angeli (ZARPELON et al., 2013), anetol (RITTER et al, 2013). Além disso, já demonstrou-se a participação da caspase-1 na gênese da resposta inata induzida pela carragenina (CUNHA et al., 2010), bem como outras citocinas, conforme mencionado anteriormente.

Tem sido demonstrado a participação da IL-1β na indução da hiperalgesia inflamatória mediada por prostanóides, e modelos de inflamação pela administração de carragenina e lipolissacarídeo (LPS) (FERREIRA et al., 1988). A hiperalgesia mecânica induzida por carragenina (Cg) e LPS é reduzida em 50% pelo pré-tratamento local com anticorpo anti-IL-1β (CUNHA et al., 1992). Conhecendo o papel da IL-1β na hiperalgesia mecânica e modelos de inflamação inata avaliamos o papel da IL-33, uma citocina da mesma família, na hipernocicepção induzida pela carragenina, um modelo de inflamação inata amplamente estudado (Anexo 1).

1.3. Dor neuropática

A Associação Internacional para o Estudo da Dor (IASP) propõe que a dor neuropática é uma dor que surge diretamente de uma lesão ou de uma doença do sistema somatossensorial. A dor neuropática é uma dor causada pela injúria do sistema nervoso, dependendo do local do dano, a dor pode ser classificada como periférica ou central.

Existem muitas causas da dor neuropática periférica, tais como infecções, trauma, anormalidades metabólicas, quimioterapia, cirurgia, radiação, compressão do nervo e infiltração tumoral (DWORKIN et al., 2003). A dor após a injúria do nervo é caracterizada por dor espontânea (independente do estímulo), alodinea e hiperalgesia (DWORKIN et al., 2003).

A dor neuropática afeta a qualidade de vida de milhões de pessoas atualmente, e frequentemente são acompanhadas pela resistência às drogas analgésicas mais utilizadas. Nas ultimas décadas tem sido sugerido que a injúria do nervo produz alterações celulares e moleculares que resultam em plasticidade neuronal e reorganização no microambiente do local da lesão, gânglio da raiz dorsal e medula espinal (INOUE et al., 2007).

A injúria do nervo provoca uma reação de células imunes e da glia em diferentes locais. Macrófagos e células de Schwann facilitam a degeneração walleriana culminando na separação de parte do axônio em relação ao corpo do neurônio devido à lesão (SCHOLZ & WOOLF, 2007). A primeira citocina que aparece aumentada em células da Schwann horas após a injúria é o TNF-α (CAMPANA et al., 2006). Além disso, alguns estudos recentes relacionaram os receptores TLRs ao desenvolvimento da dor neuropática. Os receptores TLR pertencem a uma família de 12 receptores conservados durante a evolução, cuja ligação pode resultar na ativação do fator de transcrição NFκB, aumento da produção de interferons e expressão de citocinas pró-inflamatórias. Em camundongos TLR2$^{-/-}$ (KIM et al., 2007) e TLR4$^{-/-}$ (TANGA et al., 2005), e em ratos tratados com antisense oligodeoxinucleotídeos anti-TLR4 (TANGA et al., 2005) foi observada redução da ativação da micróglia, da produção de citocinas pró-inflamatórias e dor neuropática comparado com animais controle após a lesão periférica de nervo.

O aumento da síntese e liberação de citocinas como IL-1β, IL-6 e TNF-α pode modular a atividade neuronal e induzir descargas de potencial de ação. Corroborando a participação de citocinas nesse fenômeno, a superexpressão do IL-1ra (antagonista de receptor de IL-1) ou deleção do IL-1R1 (receptor ativado pela IL-1β) inibe o disparo espontâneo de neurônios sensoriais (descargas ectópicas) (WOLF et al., 2006). Além disso, o bloqueio dos sinais induzidos pela IL-1β ou IL-6 atenua a hiperalgesia em modelo de neuropatia (ARRUDA et al., 2000; WOLF et al., 2006). Por sua vez, o TNF-α tem efeito direto (via seu receptor) aumentado nos neurônios sensoriais após lesão do nervo, pois tanto as fibras nervosas lesionadas e não lesionadas da vizinhança tornam-se mais sensíveis a essa citocina (SCHAFERS et al., 2003). Além da participação de uma via envolvendo a ativação dos receptores TNFR1 e MAP quinases como a p38, o TNF-α também atua aumentando a densidade de canais de sódio resistentes a tetrodotoxina cuja ativação está relacionada com a hiperagelsia (JIN & GEREAU, 2006).

Em modelos de lesão periférica de nervo ocorre a ativação de receptores TLR-4 em micróglia e como resultado ocorre a produção de IL-18 de maneira dependente da MAP quinase p38. Por sua vez, a IL-18 liberada pela micróglia ativa astrócitos induzindo a ativação de NFκB e consequentemente a produção de outras citocinas que atuam sensibilizando o nociceptor (MIYOSHI et al., 2008). Previamente também demonstramos que a IL-18 é uma citocina hiperalgésica em modelos de inflamação periférica (VERRI et al., 2004; 2007; 2008). A IL-18 é uma citocina da família da IL-1 que também inclui o seu mais recente membro, a IL-33 (SCHMITZ et al., 2005). A IL-33 induz a hiperalgesia periférica via produção de TNF-α e IL-1β (VERRI et al., 2008). Essas citocinas também são importantes na dor neuropática (NADEAU et al., 2011).

Além disso, SCHMITZ e colaboradores (2005), demonstraram que ocorre a expressão de RNAm para IL-33 na medula espinal, bem como na micróglia (HUDSON et al., 2008). Tanto a medula espinal, quanto as células da glia participam na neuropatia (TANGA et al., 2005), bem como, MAP quinases e NFκB (TANGA et al., 2005; MIYOSHI et al., 2008). Sendo assim, propôs-se a investigar se a IL-33 é um mediador importante na medula espinal e células da glia para o aumento da sensibilidade mecânica na neuropatia induzida pela lesão periférica de nervo.

1.4. Papel das células da glia nas respostas inflamatórias

As células da glia são um conjunto de células não neuronais do sistema nervoso, de 10 a 50 vezes mais numerosas que os neurônios. São quatro principais grupos: astrócitos, oligodendrócitos e micróglia, no sistema nervoso central (cérebro e medula espinal) e as células satélite, no sistema nervoso periférico.

Dentre os quatro principais grupos, destacamos os astrócitos e a micróglia. Os astrócitos são em número e volume as células mais abundantes, e correspondem a quase 50% de toda a glia (ALDSKOGIUS & KOSLOVA, 1998). São células de formato estrelado e apresentam filamentos intermediários constituídos pela proteína fibrilar ácida da glia (GFAP). Estas células são capazes de se ligar em até 6 corpos celulares de uma única vez, e permitem a ligação neurônio à capilares sanguíneos. Já a micróglia são células pequenas e alongadas, com prolongamentos curtos e irregulares. São conhecidas como os fagócitos do SNC e são importantes na inflamação, reparação e também na secreção de citocinas (GAO & JI, 2010).

Em condições fisiológicas, os astrócitos e as células satélite exercem suas funções ativamente, enquanto que a micróglia permanece em estado de repouso. Entretanto, quando ocorre o aumento de cálcio intracelular, liberação de citocinas e/ ou outros mediadores inflamatórios, bem como a expressão de receptores na superfície de neurônios e outras células da glia, a micróglia torna-se ativa (RANSOHOFF & CARDONA, 2010).

O astrócito tem uma expressão basal da proteína citoplasmática fibrilar ácida da glia (GFAP), que é aumentada proporcionalmente à ativação da célula. A Iba-1 (molécula adaptadora da ligação ao cálcio ionizado 1) é uma proteína de canal iônico especificamente expressa em macrófagos e micróglia, e tem sua expressão aumentada mediante ativação (GAO & JI, 2010).

Geralmente na dor neuropática, acredita-se que as células gliais da medula espinal estejam envolvidas no aumento e manutenção da dor. Essas células podem ser responsáveis pela liberação de neuromoduladores, tais como citocinas pro-inflamatórias, quimiocinas e fatores de crescimento (WATKINS & MAIER, 2002). Mas ainda não está totalmente claro como ocorre a ativação das células da glia, ou mesmo como elas atuam para a indução e/ou manutenção da dor.

Alguns mecanismos vem sendo sugeridos para ativação glial durante a dor crônica. As fibras nociceptivas, uma vez ativadas pela liberação de neurotransmissores (ATP, glutamato e substância P), são capazes de sensibilizar o neurônio nociceptivo secundário, bem como, ativar as células da glia. Uma vez ativada, a glia libera principalmente citocinas pró-inflamatórias, dentre as quais se destacam IL-1β e TNF-α. HUDSON e colaboradores (2008) demonstraram que a IL-33 é produzida após a estimulação de astrócitos com LPS e ATP. Esse estudo ilustra que a IL-33 pode ter um papel importante na imunidade inata via ativação das células gliais. Os mesmos autores acreditam que essas citocinas podem ser a ligação entre o dano tecidual, ativação das células da glia e consequente manutenção da dor.

Recentemente ZHAO e colaboradores (2013), avaliaram a participação da IL-33 em um modelo de dor no câncer, caracterizado por hiperalgesia mecânica, térmica e dor espontânea. Os autores demonstraram que a IL-33 está localizada tanto na micróglia quanto nos astrócitos na medula espinal, mas não nos neurônios. A IL-33 uma vez ligada ao seu receptor poderia resultar na ativação de proteínas adaptadoras como MyD88, fatores de transcrição como o NFκB, e MAP quinases.

Acredita-se que a MAP quinase ERK seja necessária para ativação das células da glia e desenvolvimento da hiperalgesia na dor do câncer (WANG et al., 2012), entretanto, o papel da IL-33 na ativação de MAP quinases e consequente ativação de células da glia ainda necessita de mais estudos.

As citocinas como TNF-α (ZHOU et al., 2010) uma vez liberados pelas células da glia, na medula ou gânglio da raiz dorsal, sensibilizam a fibra, potencializando e cronificando o processo doloroso. Já o papel da IL-33 na ativação de células da glia e consequente desenvolvimento da dor neuropática ainda é desconhecido.

1.5. IL-33

O receptor para IL-33 foi demonstrado pela primeira vez em 1989. Este foi originalmente descrito como um soro indutor da secreção de proteínas em fibroblastos murinos (TOMINAGA, 1989). Além desse estudo, BERGES e colaboradores (1994) demonstraram a existência de um único gene que expressa proteínas solúveis e de membrana, além disso, a expressão de cada proteína é regulada independente, sendo nomeadas como Fi1-IS e Fit-IM, os quais são pertencentes à família da IL-1 e podem regular as funções fisiológicas dessa citocina.

Outro estudo importante detectou que o ST2, também chamado de DER4, Fit-1 ou T, estava presente na superfície de células estáveis, que é expresso em células Th2, mas não em células Th1. O gene foi detectado em cultura de células hematopoiéticas da medula óssea de camundongos adultos, sendo expresso em resposta a estimulação por IL-4, mas não por interferon (IFN)-γ. O anticorpo contra esse receptor aumentou a resistência à infecção por *Leishmania major* em animais BALB/c, e exacerbou a artrite induzida por colágeno, em camundongos DBA. Assim, os autores concluíram que o receptor ST2L, poderia ser um importante alvo na superfície celular durante as respostas imunes (XU et al., 1998).

Anteriormente chamada de NF-HEV (fator nuclear derivado de vênulas endoteliais altas), Baekkevold em 2003, determinou que esse fator poderia ser necessário para o controle do endotélio venular. Dois anos depois, Schmitz e colaboradores (2005), redescobriram a existência da IL-33, uma citocina da família da IL-1 que sinaliza via receptor ST2 e induz citocinas relacionadas à resposta Th2, via ativação do fator de transcrição κB (NFκB) e MAP quinases (SCHMITZ et al., 2005). A família da IL-1 contem 11 membros: IL-1α, IL-1β, agonista do receptor da

IL-1 (IL-1Ra), IL-18, IL-1F5, IL-1F6, IL-1F7, IL-1F8, IL-1F9, IL-1F10 e IL-33. Citocinas da família da IL-1, geralmente necessitam de um precursor protéico para liberação da sua forma ativa. Para a atividade da IL-18 é necessária uma clivagem pela enzima ICE (enzima conversora de interleucina 1-β), a qual cliva a pró-IL-18 em IL-18 ativa. Schmitz e colaboradores (2005) relataram que a IL-33 necessitaria de uma clivagem pela caspase-1, para liberação de sua forma ativa. Mais tarde, foi detectado que apesar de pertencerem à mesma família, os mecanismos envolvidos de ativação são diferentes, já que caspase-1 é capaz de clivar IL-1β e IL-18 durante a apopose, mas inativa a IL-33 (CAYROL et al., 2009). A inativação da IL-33, provavelmente ocorre para prevenir a liberação desta durante um processo de morte celular que não requer resposta inflamatória.

Ao contrário do que ocorre durante a apoptose, no processo de dano ou estresse celular, caracterizados pelos processos de necrose e recrutamento de neutrófilos, a atividade da IL-33 pode aumentar cerca de 10 vezes. Dessa forma, tem-se sugerido a ação da IL-33 como um “sinal de perigo”, bem como a alarmina do grupo Box de alta mobilidade (HMGB)-1. Os mecanismos pelos quais ocorre a liberação da IL-33 ainda não foram completamente elucidados, entretanto, sabe-se que o dano celular leva à liberação da IL-33 (LEFRANÇAIS & CAYROL, 2012).

A IL-33 é encontrada em células endoteliais, células epiteliais (trato respiratório, pulmão, estômago, trato digestivo vagina e pele), medula espinal, fibroblastos, órgãos linfóides (baço, linfonodo) e no cérebro (SCHMITZ et al., 2005). A IL-33 se liga ao receptor ST2 e a uma proteína acessória IL-1RAcP, sua ação pode ser autócrina, parácrina ou endócrina. Além disso, existe a forma solúvel do receptor (sST2) que foi amplamente encontrada em fibroblastos, macrófagos e monócitos estimulados com LPS, TNF-α, IL-1β, bem como em clones ativados de células Th2. Altas concentrações do receptor solúvel para ST2 foi encontrado em várias doenças com padrão Th2, incluindo o lúpus eritematoso, asma e condições inflamatórias independente da resposta Th2, como choque séptico e trauma (TRAJKOVIC et al., 2004).

A maioria dos trabalhos difundidos até o momento, evidenciam a IL-33 como uma citocinas Th2, entretanto, alguns dados demonstram que o tratamento com sST2 inibe a severidade da artrite (LEUNG et al., 2004), a IL-33 medeia a inflamação em modelo de artrite reumatóide (VERRI et al., 2008), detectou-se a expressão de RNA mensageiro para IL-33 em amostras de tecido sinovial de pacientes com artrite reumatoide (CARRIERE et al., 2007). Dessa forma, existem evidências de que a IL-33 possa também mediar respostas Th1, assim crescem as evidências que a demonstram como uma citocina pleiotrópica.

Nosso grupo foi o primeiro a demonstrar o papel da IL-33 na dor. Em 2008, Verri e colaboradores relataram que a IL-33 é um importante mediador na dor induzida pela hipernocicepção na artrite induzida por antígeno. As diferentes famílias de citocinas ligam-se a receptores específicos, desencadeando a ativação de uma série de vias de sinalização, por isso, avaliar a participação de uma nova citocina em modelo de dor neuropática, como a IL-33, pode cooperar para o entendimento dos processos inflamatórios.

2. Objetivos

2.1. Objetivo geral

Avaliar a participação da IL-33 e os mecanismos espinais envolvidos na hiperalgesia induzida pelas carragenina e injúria por constrição crônica do nervo ciático em camundongos, com enfoque na participação das vias PI3K/AKT, mTOR, MAP quinases, NFκB e células da glia.

2.2. Objetivos Específicos

2.2.1. Resposta inflamatória inata – carragenina

- Avaliar se a carragenina induz a expressão de RNAm para IL-33 e ST2 e produção de IL-33;

- Avaliar se a IL-33 participa da hiperalgesia mecânica, edema e atividade de mieloperoxidase induzidos pela carragenina;

- Avaliar se a IL-33 mimetiza a hiperalgesia, edema e atividade de mieloperoxidase;

- Utilizar ferramentas farmacológicas a fim de avaliar se o recrutamento leucocitário (fucoidina), TNF-α (infliximab), CXCL1 (anticorpo contra CXCL1), IL-1 (antagonista do receptor da IL-1), endotelinas, receptor ETA (clazosentan) e ETB (BQ788) e COX (indometacina) são importantes na hiperalgesia induzida pela IL-33;

- Avaliar se a produção de TNFα, CXCL1 e IL-1β após a administração de carragenina é dependente de IL-33;

- Avaliar a produção de IL-10 após a administração de carragenina é dependente de IL-33;

- Analisar se a IL-33 pode amplificar a resposta induzida pela carragenina, utilizar-se-á doses submáximas de IL-33 e carragenina a fim de verificar o efeito sinérgico.

2.2.2. Dor neuropática induzida pela injúria por constrição crônica

- Avaliar se a deficiência da IL-33 é importante no modelo de injúria induzido por constrição crônica do nervo ciático de camundongos;

- Avaliar se a IL-33 mimetiza a hiperalgesia mecânica induzida pela injuria por constrição crônica, bem como, verificar se é dependente de IL-1β e TNF-α;

- Verificar se a hiperalgesia induzida pela administração i.t. de IL-33 e a injúria por constrição crônica é dependente das vias PI3K, mTOR, MAP kinases (p38, ERK and JNK) e NFκB, através da administração de inibidores;

- Verificar a ativação espinal das vias PI3K, AKT, mTOR, MAP kinases e NFκB pelo método de western blot;

- Verificar se os tratamentos com minociclina e fluorocitrato inibem a hiperalgesia induzida pela IL-33 e pela injúria por constrição crônica do nervo ciático de camundongos;

- Verificar a ativação espinal de GFAP e Iba-1 após a administração i.t. de IL-33 e a injúria por constrição crônica do nervo ciático.

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4. Anexos

4.1. Anexo 1

IL-33/ST2 signalling contributes to carrageenin-induced innate inflammation and inflammatory pain: role of cytokines, endothelin-1 and prostaglandin E_2

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BACKGROUND AND PURPOSE

IL-33 signals through ST2 receptors and induces adaptive and innate inflammation. IL-33/ST2 is involved in adaptive inflammation-induced pain. Here, we have investigated the contribution of IL-33/ST2-triggered mechanisms to carrageenin-induced innate inflammation.

EXPERIMENTAL APPROACH

Carrageenin- and IL-33-induced inflammatory responses were assessed in BALB/c- (WT) and ST2-deficient ($^{-/-}$) mice as follows: oedema (plethysmometer), myeloperoxidase activity (colorimetric assay), mechanical hyperalgesia (electronic version of von Frey filaments), cytokine levels (ELISA), PGE_2 (RIA), mRNA expression (quantitative PCR), drug treatments targeting leukocyte recruitment (fucoidin), TNF-α (infliximab), CXCL1 (antibody to CXCL1), IL-1 (IL-1ra), endothelin ET_A (clazosentan) and ET_B (BQ788) receptors and COX (indomethacin).

KEY RESULTS

Carrageenin injection increased ST2 and IL-33 mRNA expression and IL-33 production in paw skin samples. Carrageenin-induced paw oedema, hyperalgesia and myeloperoxidase activity were reduced in $ST2^{-/-}$ compared with WT mice, effects mimicked by IL-33 injection in the paw. Furthermore, IL-33-induced hyperalgesia was reduced by fucoidin suggesting a role for recruited leukocytes in its hyperalgesic effect. IL-33-induced hyperalgesia in naïve mice was reduced by treatments targeting TNF, CXCL1, IL-1, endothelin receptors and COX while carrageenin-induced ST2-dependent TNF-α, CXCL1, IL-1β, IL-10 and PGE_2 production and preproET-1 mRNA expression. Combining IL-33 and carrageenin at doses that were ineffective as single treatment induced significant hyperalgesia, oedema, myeloperoxidase activity and cytokine production in a ST2-dependent manner.

CONCLUSIONS AND IMPLICATIONS

IL-33/ST2 signalling triggers the production of inflammatory mediators contributing to carrageenin-induced inflammation. These data reinforces the importance of IL-33/ST2 signalling as a target in innate inflammation and inflammatory pain.

Abbreviations

ET-1, endothelin-1; i.pl., intraplantar; IL-1ra, IL-1 receptor antagonist

Introduction

IL-33 is a member of the IL-1 family of cytokines, which signals through the ST2/IL-1RAcP receptor complex (Schmitz *et al.*, 2005; Ali *et al.*, 2007). IL-33 seems to be an important therapeutic target or therapy in inflammatory conditions such as anaphylactic shock, septic shock, atherosclerosis, UV radiation, asthma, hepatitis and rheumatoid arthritis (Schmitz *et al.*, 2005; Verri *et al.*, 2008; 2010; Pushparaj *et al.*, 2009; Alves-Filho *et al.*, 2010; Arshad *et al.*, 2011; Byrne *et al.*, 2011). These roles of IL-33 are related to the activation of endothelial cells, mast cells, lymphocytes, macrophages, eosinophils and neutrophils (Schmitz *et al.*, 2005; Pecaric-Petkovic *et al.*, 2009; Alves-Filho *et al.*, 2010; Verri *et al.*, 2010).

IL-33 was initially recognized as a Th2 cytokine (Schmitz *et al.*, 2005). Further investigation of the biology of IL-33/ST2 signalling showed that it is an important component in Th1/Th17 and innate inflammation. Thus, IL-33 mediates antigen-induced arthritis dependent on TNF-α, IL-1β and IFN-γ (Verri *et al.*, 2008; 2010) and IL-17 production (Xu *et al.*, 2008). IL-33 also amplified Th1 and Th2 responses by acting on basophils, Th2 lymphocytes, INKT and NK cells (Smithgall *et al.*, 2008). In support of a role of IL-33 in innate inflammation, glia secreted IL-33 in response to LPS (Hudson *et al.*, 2008) and IL-33 administration reduced the systemic inflammatory response induced by bacterial products in a model of sepsis (Alves-Filho *et al.*, 2010). IL-33 also increased expression of LPS receptor components resulting in enhanced cytokine production, suggesting a LPS self-regulatory feedback through IL-33 (Espinassous *et al.*, 2009). Furthermore, IL-33 deficiency resulted in reduced papain-induced lung innate inflammation and dextran-induced colitis accompanied by T-cell-independent epithelial damage (Oboki *et al.*, 2010). Moreover, IL-33 induced cytokine production by type 2 innate immune cells such as natural helper cells, nuocytes, and innate helper 2 cells (Kouzaki *et al.*, 2011; Kim *et al.*, 2012). However, ST2 deficiency increased innate and acquired Th1 and Th2 immunity in response to a murine mammary carcinoma (Jovanovic *et al.*, 2011). Therefore, the functions of IL-33 are not completely predictable.

Carrageenin is an extensively used and accepted model of inflammation. In general, it is injected in the paw to evaluate oedema and hyperalgesia, but it can also be injected in other cavities such as air pouch, peritoneal and pleural cavities to evaluate leukocyte recruitment, mainly of neutrophils, and production of chemotactic mediators (Cunha *et al.*, 2005; Valério *et al.*, 2007). The intraplantar injection of carrageenin induces mechanical hyperalgesia in mice by triggering a cytokine cascade initiated by TNF-α and CXCL1 production, which induce IL-1β-dependent PGE_2 production. In turn, PGE_2 sensitizes the nociceptor, which can be detected as mechanical hyperalgesia (Cunha *et al.*, 2005). Furthermore, endothelin-1 (ET-1) acting on ET_A and ET_B receptors mediates carrageenin-induced mechanical hyperalgesia (Baamonde *et al.*, 2004; receptor nomenclature follows Alexander *et al.*, 2011) and ET-1-induced hyperalgesia depends on PGE_2 production (Verri *et al.*, 2007). Recruited neutrophils also contribute to carrageenin-induced mechanical hyperalgesia by producing PGE_2 (Cunha *et al.*, 2008).

As IL-33 mediates mechanical hyperalgesia (Verri *et al.*, 2008) and neutrophil recruitment (Verri *et al.*, 2010) in adaptive inflammation, and is involved in innate inflammation (Hudson *et al.*, 2008; Espinassous *et al.*, 2009; Alves-Filho *et al.*, 2010; Oboki *et al.*, 2010; Zhang *et al.*, 2011), we have investigated whether IL-33/ST2 contributed to carrageenin-induced innate inflammation and inflammatory pain and the underlying mechanisms. Furthermore, because IL-33 potentiated antigen-induced production of inflammatory cytokines (Andrade *et al.*, 2011), we also assessed whether IL-33 could act in synergy with carrageenin to induce paw inflammation and hyperalgesia.

Methods

Animals

Sex matched BALB/c (WT, $ST2^{+/+}$) and BALB/c background ST2-deficient ($^{-/-}$) mice (Brint *et al.*, 2004), 20–25 g were bred in the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil. All animal care and experimental procedures complied with the International Association for the Study of Pain guidelines and approved by the Ethics Committee of the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 908 animals were used in these experiments.

Electronic pressure meter test

The mechanical hyperalgesia test (Cunha *et al.*, 2004) consisted of evoking a hindpaw flexion reflex with a handheld force transducer (electronic anaesthesiometer; IITC Life Science, Woodland Hills, CA, USA) adapted with a 0.5 mm^2 polypropylene tip. The results are expressed as the differential (Δ) withdrawal threshold (in g) calculated by subtracting the mean measurements at indicated time points after stimulus from the zero-time mean measurements. Withdrawal threshold was 8.6 ± 0.5 g (mean ± SEM, $n = 40$) before injection of the hyperalgesic agents.

Paw oedema test

The volume of the paw was measured with a plethysmometer (Ugo Basil, Comerio, VA, Italy) before (V0) and at indicated time points after (VT) the intraplantar (i.pl.) stimulus with carrageenan (3 or 100 μg diluted in 25 μL of saline per paw) (Valério *et al.*, 2007). The amount of paw swelling was determined for each mouse and the difference between VT and V0 was taken as the oedema value (oedema mm^3 per paw).

Myeloperoxidase activity

The myeloperoxidase activity of paw homogenates was used to evaluate the migration of leukocytes to the hind paw skin of mice. It consists of a kinetic colorimetric assay (Bradley *et al.*, 1982) with modifications (Casagrande *et al.*, 2006). The results were presented as the myeloperoxidase activity (number of neutrophils 10^4 per paw).

Cytokine measurement

Animals were terminally anaesthetized (1.5% isoflurane; Abbott, (Abbott Park, IL, USA) and the plantar skin tissues removed and homogenized in 500 µL of buffer containing protease inhibitors. Concentrations of TNF-α, IL-1β, CXCL1, IL-10 (BD Bioscience, San Jose, CA, USA) and IL-33 (eBioscience, San Diego, CA, USA) were determined by ELISA using paired antibodies.

Real-time PCR

Mice were killed 2 h after i.pl. injection of carrageenin and the plantar cutaneous hind paw tissues harvested. Samples were homogenized in Trizol reagent, and total RNA was extracted using the SV Total RNA Isolation System (Promega Biosciences, Fitchburg, WI, USA). Quantitative PCR (qPCR) was performed in an ABI Prism 7500 Sequence Detection System using the SYBR-green fluorescence (Applied Biosystems, Grand Island, NY, USA). The primers were previously described (Verri *et al.*, 2008).

Determination of PGE$_2$ production

Two hours after i.pl. injection of carrageenin, paw skin tissue samples were collected in 0.5 mL of a mixture of acetone: 1 M HCl: water (10:1:5, v : v : v). After homogenizing with a turrax, the samples were centrifuged (20 min at 2000 *g* at 4°C) and the supernatant decanted before drying the pellet in a centrifugal evaporator at 37°C. The pellet was reconstituted in 500 µL of Tris/HCl buffer (10mM Tris, pH adjusted with HCl to 8.0). The concentrations of PGE$_2$ were determined by RIA (Amersham, Pittsburgh, PA, USA) (Verri *et al.*, 2007).

Experimental protocols

WT mice received intraplantar (i.pl., subcutaneous in the hind paw) injection of carrageenin (100 µg per paw) or saline. Samples of the paw skin were collected at 2 h for qPCR analysis for ST2, IL-33 and preproET-1 mRNA expression. IL-33 levels were determined 0.5, 1, 3 and 5 h after carrageenin by ELISA. Mechanical hyperalgesia, oedema and myeloperoxidase activity were evaluated at indicated time points after carrageenin (3 or 100 µg-per paw) or IL-33 (3, 30, 100 and 300 ng-per paw) injection in WT and ST2$^{-/-}$ mice. In other experiments, WT mice were treated with fucoidin (20 mg·kg^{-1}, i.v, 15 min), infliximab (anti-TNF-α antibody, 10 mg·kg^{-1}, i.p, 48 h and 60 min before stimuli), anti-CXCL1 antibody (αCXCL1; 700 ng·mice^{-1}, i.p. injection, in saline, 30 min before stimulus), control IgG (same treatment protocol as for infliximab or αCXCL1) or IL-1 receptor antagonist (IL-1ra; 30 mg·kg^{-1}, i.v., 15 min) before IL-33 administration (100 or 300 ng-per paw) followed by evaluation of mechanical hyperalgesia and myeloperoxidase at indicated time points. TNF-α, CXCL1, IL-1β, IL-10 and PGE$_2$ levels in paw skin samples were determined by ELISA or RIA at 2 h or indicated time points after carrageenin injection. In the last series of experiments, mice were treated with clazosentan (10 mg·kg^{-1}, s.c., 15 min), BQ 788 (30 nmol per paw, 30 min) or indomethacin (5 mg·kg^{-1}, i.p., 40 min) before i.pl. injection of IL-33 (100 ng- per paw) or saline. Mechanical hyperalgesia was evaluated 3 and 5 h after. To determine the synergism between IL-33 and carrageenin, mice received single treatments with carrageenin (3 µg per paw) or IL-33 (3 ng-per paw) or a combination of both followed by mechanical hyperalgesia, oedema and MPO activity determination at indicated time points. The same protocol was used to determine the production of TNF-α and IL-1β in WT (ST2$^{+/+}$) and ST2$^{-/-}$ mice. The doses of reagents used *in vivo* were chosen based on previous reports (Cunha *et al.*, 2005; 2008; Verri *et al.*, 2006; 2008; 2009; 2010; Pinto *et al.*, 2010). Drug treatments or genetic deficiency did not affect the baseline response of the mice to mechanical stimulation (data not shown).

Data analyses

Results are presented as means ± SEM; each experiment was performed at least twice. Two-way ANOVA was used to compare the groups and doses at all times when the parameters were measured at different times after the stimulus injection. The analysed factors were treatments, time and time versus treatment interaction. One-way ANOVA followed by Bonferroni's *t*-test was performed for each time. Comparison of two groups was performed using *t*-test. $P < 0.05$ was considered significant.

Materials

The following materials were obtained from the sources indicated: anti-CXCL1 antibody (Peprotech, Rocky Hill, NJ, USA), isotype control IgG (R&D Systems, Minneapolis, MN, USA), BQ 788 (Tocris Bioscience, Ellisville, MO, USA), carrageenin (FMC Corporation (Philadelphia, PA, USA), clazosentan (Actelion Pharmaceutical Ltd., Allschwil/Basel, Switzerland), fucoidin (Sigma Chemical, St Louis, MO, USA), IL-1ra (National Institute of Biological Standards and Control, Hertfordshire, UK), indomethacin (Prodome, Campinas, Brazil), infliximab (Remicade, Merck & Co., Whitehouse Station, NJ, USA). Recombinant human IL-33 was generated as previously described (Komai-Koma *et al.*, 2007).

Results

Carrageenin induces IL-33 and ST2 mRNA expression and IL-33 production in the paw skin

Carrageenin (100 µg-per paw) or saline (25 µL) was injected in WT mice and after 2 h paw skin samples were collected for qPCR analysis. Carrageenin induced a significant increase of ST2 (Figure 1A) and IL-33 (Figure 1B) mRNA expression. In agreement, carrageenin induced a significant increase of IL-33 production after 0.5–5 h (Figure 1C).

Reduction of carrageenin-induced mechanical hyperalgesia, oedema and neutrophil recruitment in ST2$^{-/-}$ mice

Carrageenin (100 µg per paw) or saline was injected in WT and ST2$^{-/-}$ mice, and mechanical hyperalgesia (Figure 2A) and oedema (Figure 2B) were evaluated 1–5 h after carrageenin. At 5 h, mice were killed and myeloperoxidase activity was determined in paw skin samples (Figure 2C). Carrageenin-induced mechanical hyperalgesia between 1 and 5 h, and oedema between 0.5 and 5 h in WT mice compared with vehicle group (Figure 2A and B, respectively). On the other hand, ST2$^{-/-}$ mice presented reduced hyperalgesia and oedema at the same time points (Figure 2A and B, respectively). The myeloperoxidase activity was increased by carrageenin injection in WT mice compared with the saline group and this increase was reduced in ST2$^{-/-}$ mice (Figure 2C).

IL-33 injection mimics carrageenin-induced inflammatory responses in a ST2-dependent manner in naïve mice

Mice received IL-33 (30–300 ng-per paw) and mechanical hyperalgesia (Figure 3A) and oedema (Figure 3B) were evaluated after 0.5–5 h. At 5 h samples of paw skin tissue were collected for myeloperoxidase activity determination (Figure 3C). The dose of 30 ng-per paw of IL–33-induced mechanical hyperalgesia between 1 and 5 h compared with vehicle group (Figure 3A), and the doses of 100 and 300 ng-per paw of IL-33 induced significant hyperalgesia compared with vehicle and the dose of 30 ng-per paw between 0.5 and 5 h (Figure 3A). IL-33 induced significant paw oedema between 0.5 and 3 h, only at 100 ng-per paw (Figure 3B). At 5 h, IL-33 induced a dose-dependent increase of myeloperoxidase activity, compared with levels after vehicle only (Figure 3C). In $ST2^{-/-}$ mice, IL-33 did not induce mechanical hyperalgesia, oedema or myeloperoxidase activity (Figure 3D–F). Thus, the injection of IL-33 mimicked carrageenin-induced inflammatory responses in WT mice, although the oedematogenic effect of IL-33, given i.pl., was clearly less than that of carrageenin. This effect was not further investigated, except for the synergism experiments presented in Figure 10.

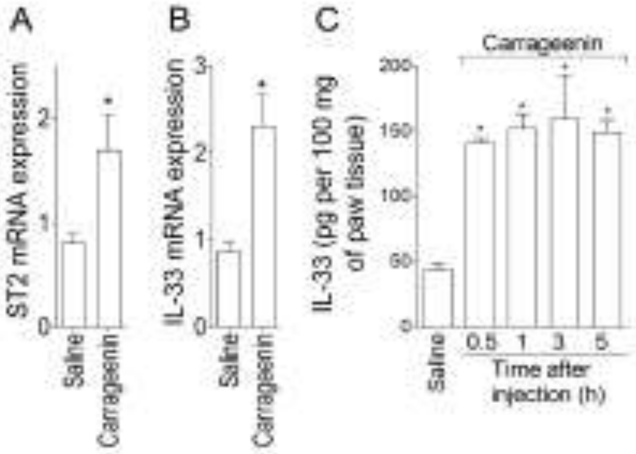

Figure 1

Carrageenin induces expression of mRNA for IL-33 and ST2 and IL-33 production in the paw skin. Mice received intraplantar (i.pl.) injection of carrageenin (100 µg per paw) or saline (25 µL), and after 2 h samples of paw skin were collected for quantitative (q) PCR analysis of ST2 (panel A) and IL-33 (panel B) mRNA expression or between 0.5 and 5 h for IL-33 determination by ELISA assay (panel C). $n = 6$ for qPCR and $n = 4$ for ELISA, representative of two separate experiments. $^{*}P < 0.05$ compared with the saline group.

Effect of fucoidin in IL–33-induced mechanical hyperalgesia in naïve mice

WT mice were treated with fucoidin (which binds to L-selectin and inhibits leukocyte recruitment; 20 mg·kg^{-1}, i.v., 15 min) before IL-33 injection (100 or 300 ng-per paw), and mechanical hyperalgesia was evaluated at 3 and 5 h and myeloperoxidase activity at 5 h (Figure 4). Two doses of IL-33 were used because 100 and 300 ng achieved maximal mechanical hyperalgesia whereas 300 ng-was needed to produce maximal myeloperoxidase activity in the paw skin. Treatment with fucoidin inhibited the mechanical hyperalgesia at 3 and 5 h (Figure 4A and C) and myeloperoxidase activity at 5 h (Figure 4B and D) induced by 100 ng-(Figure 4A and B) and 300 ng-(Figure 4C and D) of IL-33.

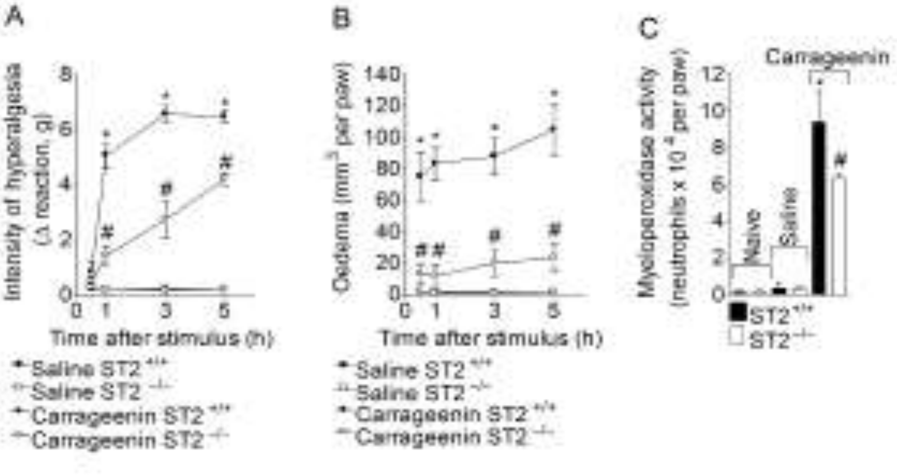

Figure 2

IL-33/ST2 mediates carrageenin-induced paw inflammation. WT ($ST2^{+/+}$) and $ST2^{-/-}$ mice received i.pl. injection of carrageenin (100 µg-per paw) or saline. Mechanical hyperalgesia (panel A) and oedema (panel B) were determined at indicated time points followed by myeloperoxidase assay at 5 h (panel C) in paw skin samples. $n = 5$, representative of two separate experiments. $^{*}P < 0.05$ compared with the saline group and $^{\#}P < 0.05$ compared with the carrageenin group.

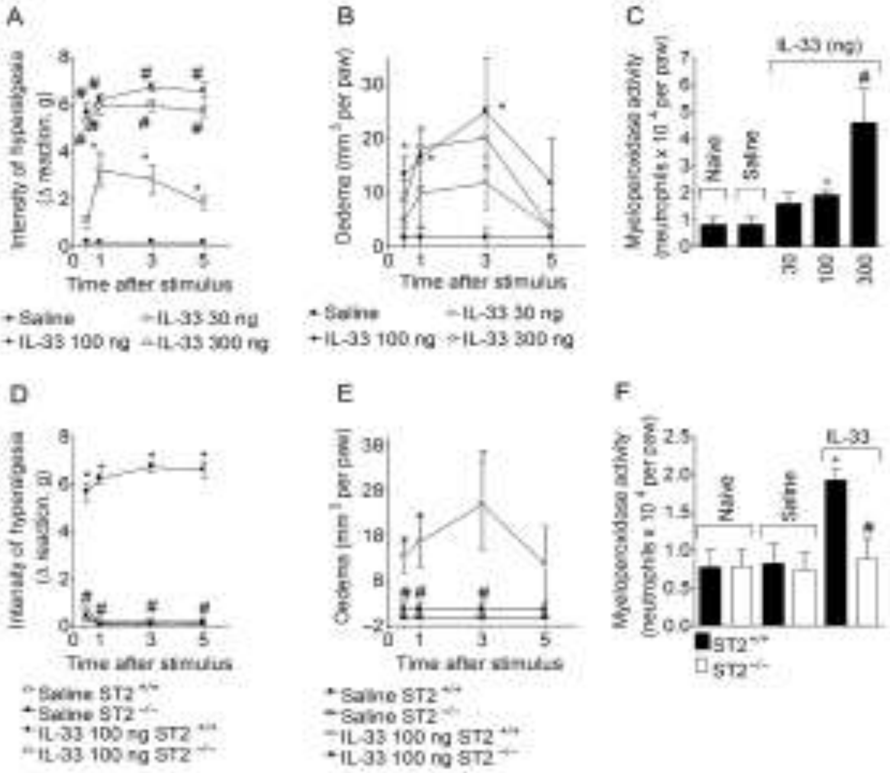

Figure 3

IL-33 induces hyperalgesia, oedema and neutrophil migration in a ST2-dependent manner in normal mice. IL-33 (30–300 ng) or saline (25 μL) was injected i.pl. in WT ($ST2^{+/+}$) mice. Mechanical hyperalgesia (panel A) and oedema (panel B) were determined at indicated time points followed by myeloperoxidase activity determination at 5 h (panel C) in paw skin samples. IL-33 (100 ng) or saline was injected i.pl. in WT and $ST2^{-/-}$ mice. Mechanical hyperalgesia (panel D) and oedema (panel E) were determined at indicated time points followed by myeloperoxidase activity determination at 5 h (panel F). $n = 6$, representative of two separate experiments. $^*P < 0.05$ compared with the saline group and $^{\#}P < 0.05$ compared with the lower dose of IL-33 or IL-33 injection in $ST2^{+/+}$ mice.

IL–33-induced mechanical hyperalgesia depends on TNF-α, CXCL1 and IL-1β in naïve mice

Mice were treated with infliximab (anti-TNF-α antibody, 10 mg·kg^{-1}, i.p. 48 h and 60 min before stimuli injection, Figure 5A and D), anti-CXCL1 antibody (αCXCL1, 700 ng·per paw, co-injection, Figure 5B and E) or IL-1ra (30 mg·kg^{-1}, i.v., 15 min, Figure 5A and D) before IL-33 injection (100 ng·per paw) and mechanical hyperalgesia was evaluated at 3 h (Figure 5A–C) and 5 h (Figure 5D–F). All treatments inhibited IL–33-induced mechanical hyperalgesia (Figure 5). Control IgG antibody (αcontrol) was used under the same treatment protocols as for infliximab (Figure 5A and D) or αCXCL1 (Figure 5B and E).

Carrageenin-induced production of TNF-α, CXCL1, IL-1β and IL-10 is decreased in ST2 deficient mice

Carrageenin (100 μg·per paw) or saline was injected in WT and $ST2^{-/-}$ mice and paw skin samples were collected after 2 h for assay of TNF-α, CXCL1 and IL-1. Carrageenin induced significant production of TNF-α (Figure 6A), CXCL1 (Figure 6B) and IL-1β (Figure 6C) in WT mice compared with the vehicle treatment, and these effects were inhibited in $ST2^{-/-}$ mice. Carrageenin also induced significant production of IL-10 at 0.5, 1, 3 and 5 h in WT mice and levels of this cytokine were also reduced in $ST2^{-/-}$ mice at 3 and 5 h after carrageenin injection (Figure 7).

ET-1 mediates IL–33-induced mechanical hyperalgesia and mRNA expression in naïve mice

Mice were treated with clazosentan (ET_A receptor antagonist, 10 mg·kg^{-1}, s.c., 30 min) or BQ788 (ET_B receptor antagonist, 30 nmol·per paw, 30 min) before IL-33 (100 ng per paw) injection (Figure 8A and B). Both clazosentan and BQ788 inhibited IL–33-induced mechanical hyperalgesia at 3 h (Figure 8A and C, respectively) and 5 h (Figure 8B and D). In agreement, carrageenin alone increased expression of mRNA for preproET-1 at 2 h after carrageenin injection in WT mice compared with the vehicle group, and this increase was lacking in $ST2^{-/-}$ mice (Figure 8E).

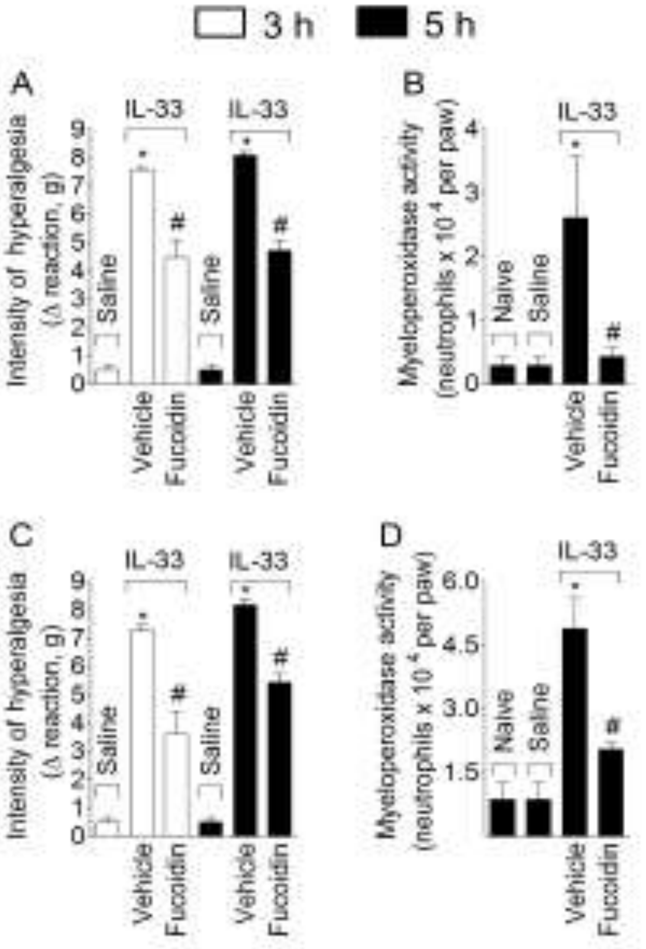

Figure 4

IL-33-induced hyperalgesia depends on neutrophil recruitment. IL-33 (100 ng – panels A and B or 300 ng – panels C and D) or saline was injected i.pl. and mechanical hyperalgesia (panel A and C) was determined at indicated time points followed by myeloperoxidase activity determination at 5 h (panel B and D) in paw skin samples. $n = 6$, representative of two separate experiments. $^*P < 0.05$ compared with the saline or naive group and $^\#P < 0.05$ compared with the IL-33 vehicle group.

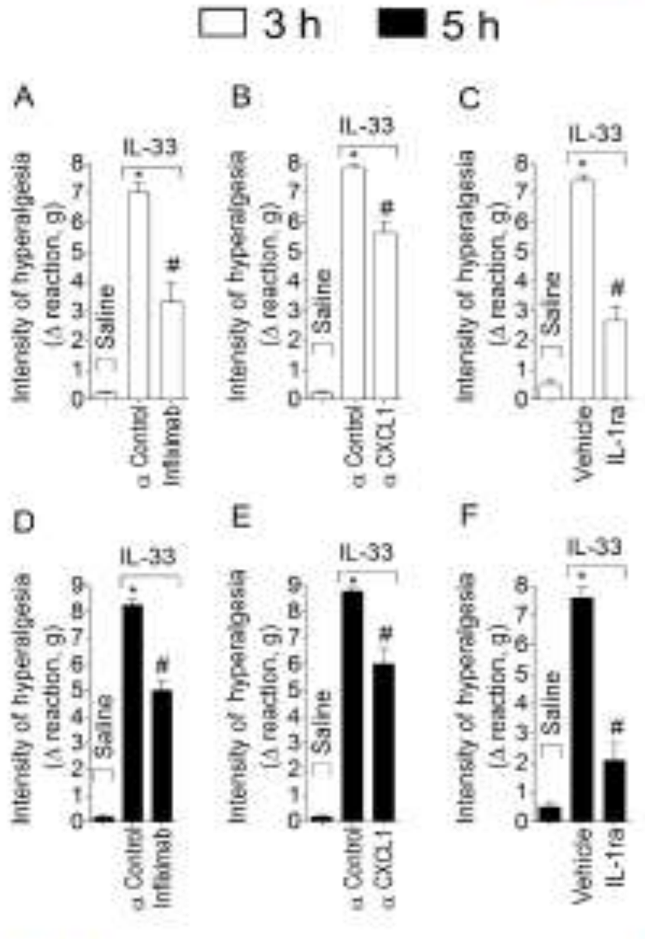

Figure 5

IL-33-induced mechanical hyperalgesia depends on TNF-α, CXCL1 and IL-1β. Mice were treated with infliximab (anti-TNF-α antibody, 10 mg·kg^{-1}, i.p. 48 h and 60 min before stimuli injection; panels A and D), anti-CXCL1 antibody (αCXCL1, 700 ng-per paw, co-injection; panels B and E), isotype IgG control antibody (αcontrol, same treatment protocol as for infliximab or αCXCL1) or IL-1 receptor antagonist (IL-1ra, 30 mg·kg^{-1}, i.v., 15 min) (panel C and F) before IL-33 i.pl. injection (100 ng-per paw). Mechanical hyperalgesia was measured 3h (panels A, B and C) and 5 h (panels D, E and F) after IL-33 injection. $n = 6$, representative of two separate experiments. $^*P < 0.05$ compared with saline group and $^\#P < 0.05$ compared with IL-33 group.

PGE$_2$ mediates IL–33-induced mechanical hyperalgesia in WT mice

Mice were treated with indomethacin (COX inhibitor, 5 mg·kg^{-1}, i.p.) 40 min before IL-33 injection (100 ng-per paw). As a result, IL–33-induced mechanical hyperalgesia was reduced at 3 h (Figure 9A) and 5 h (Figure 9B). Carrageenin also induced PGE$_2$ production in WT mice and this effect was absent in ST2$^{-/-}$ mice (Figure 9C).

IL-33 synergizes with carrageenin to induce paw inflammation and hyperalgesia

Mice received i.pl. injections of carrageenin (3 µg-per paw) alone, IL-33 (3 ng per paw) alone or a combination of IL-33 and carrageenan, in the same low doses. IL-33 and carrageenin given alone now did not induce significant mechanical hyperalgesia (Figure 10A) or oedema (Figure 10B) at 1, 3 and 5 h, or myeloperoxidase activity at 5 h. On the other hand, injection of IL-33 plus carrageenin induced significant mechanical hyperalgesia (Figure 10A), oedema (Figure 10B) and myeloperoxidase activity (Figure 10C). Furthermore, the combination of IL-33 and carrageenin also induced synergy in the production of TNF-α (Figure 10D) and IL-1β (Figure 10E), which was ST2-dependent (Figure 10D and E).

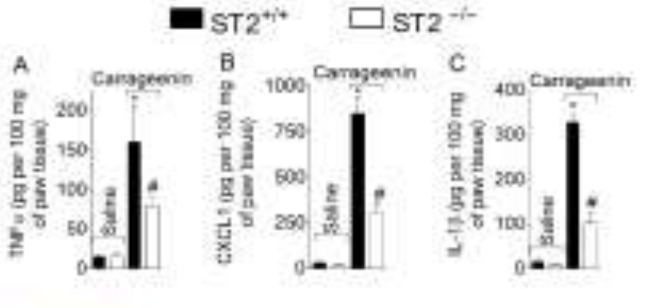

Figure 6

Carrageenin induced ST2-dependent production of TNF-α, CXCL1 and IL-1β in WT ($ST^{+/+}$) mice. Carrageenin (100 μg) was injected i.pl. and after 2 h samples of cutaneous plantar tissue were collected for determination of TNF-α (panel A), CXCL1 (panel B), and IL-1β (panel C) levels by ELISA. $n = 4$, representative of two separate experiments. $^*P < 0.05$ compared with saline group and $^\#P < 0.05$ compared with carrageenin $ST2^{+/+}$ group.

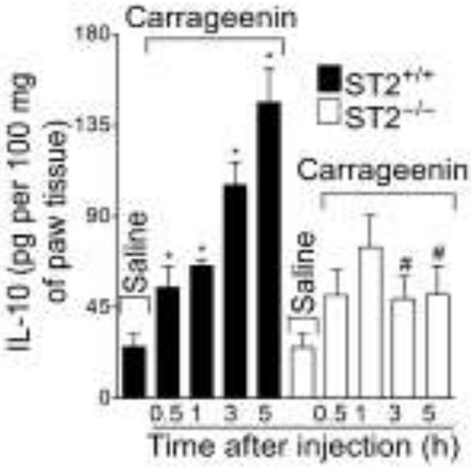

Figure 7

Carrageenin induced ST2-dependent production of IL-10 in mice. Carrageenin (100 μg) was injected i.pl. and after 0.5–5 h samples of cutaneous plantar tissue were collected for determination of IL-10 levels determination by ELISA. $n = 4$, representative of two separate experiments. $^*P < 0.05$ compared with saline group and $^\#P < 0.05$ compared with carrageenin $ST2^{+/+}$ group.

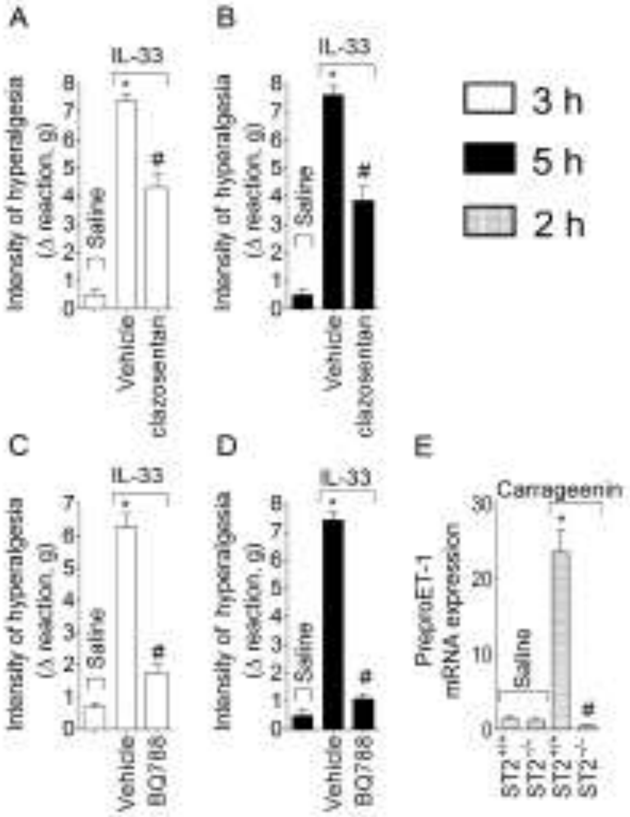

Figure 8

IL-33/ST2 mediates carrageenin-induced hyperalgesia via ET-1 acting on ET_A and ET_B receptors. Mice were treated with clazosentan (ET_A receptor antagonist, 10 mg·kg^{-1}, 30 min, panels A and B) or BQ 788 (ET_B receptor antagonist, 30 nmol·per paw, 30 min, panels C and D) before IL-33 (100 ng·per paw) injection. Mechanical hyperalgesia was measured 3 and 5 h after IL-33 injection. Carrageenin (100 μg) or saline was injected i.pl. in $ST2^{+/+}$ and $ST2^{-/-}$ mice and samples of cutaneous tissue were collected after 2 h for qPCR analysis of preproET-1 mRNA expression (panel E). $n = 6$, representative of two separate experiments. $^*P < 0.05$ compared with saline group and $^\#P < 0.05$ compared with IL-33 or carrageenin $ST2^{+/+}$ group.

Discussion and conclusions

Despite the initial description of IL-33 as a Th2 cytokine, it has become evident that it is, rather, a pleiotropic cytokine mediating Th1, Th2, Th17 and innate inflammatory responses (Schmitz *et al.*, 2005; Komai-Koma *et al.*, 2007; Smithgall *et al.*, 2008; Verri *et al.*, 2008; Xu *et al.*, 2008; Alves-Filho *et al.*, 2010; Oboki *et al.*, 2010). We have previously demonstrated the hyperalgesic role of IL-33/ST2 in a model of antigen-induced arthritis in mice (Verri *et al.*, 2008). Therefore, we hypothesised that IL-33/ST2 signalling would also be important in innate inflammation-induced hyperalgesia. The carrageenin model of innate inflammation was chosen due to its wide applicability in the study of novel analgesic and anti-inflammatory drugs. The present data demonstrate that IL-33/ST2 contributes to carrageenin-induced inflammation consisting of oedema, myeloperoxidase activity and mechanical hyperalgesia by triggering the production of other cytokines (TNF-α, CXCL1 and IL-1β), along with ET-1 and PGE_2, and also reducing the production of the anti-hyperalgesic cytokine IL-10. Furthermore, we demonstrated that injection of both IL-33 and carrageenin at doses that did not cause responses alone, induced mechanical hyperalgesia, oedema, increase of myeloperoxidase activity and cytokine production, suggesting a synergism in inducing inflammation between carrageenin-and IL-33.

IL-33 has a role in the activation of mast cells, glia cells, macrophages, epithelial and endothelial cells (Schmitz et al., 2005; Hudson *et al.*, 2008; Moussion *et al.*, 2008; Bourgeois

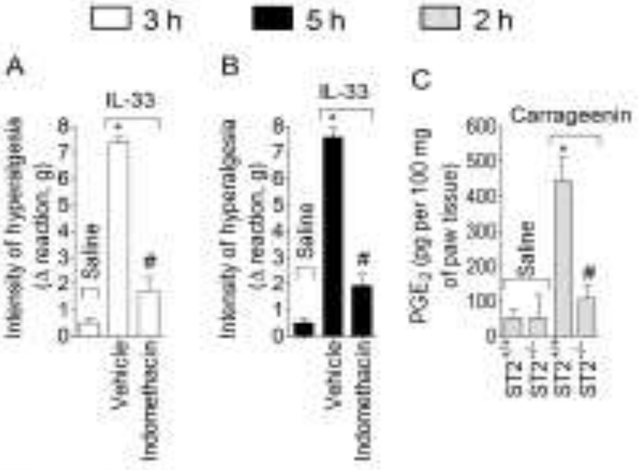

Figure 9

IL-33/ST2 mediates carrageenin-induced hyperalgesia via PGE$_2$. Mice were treated with indomethacin (COX inhibitor, 5 mg·kg^{-1}), 40 min, panels A and B) before IL-33 (100 ng·per paw) injection. Mechanical hyperalgesia was measured 3h (panel A) and 5 h (panel B) after IL-33 injection. Carrageenin (100 µg) or saline was injected i.pl. in ST2$^{+/+}$ and ST2$^{-/-}$ mice and samples of cutaneous tissue were collected after 2 h for PGE$_2$ levels determination by RIA (panel C). $n = 6$, representative of two separated experiments. *$P < 0.05$ compared with the saline group and #$P < 0.05$ compared with the IL-33 or carrageenin ST2$^{+/+}$ group.

et al., 2009; Kurowska-Stolarska *et al.*, 2009; Nelson *et al.*, 2011; Zhang *et al.*, 2011), resulting in innate inflammation. The present results add to the earlier data demonstrating that IL-33/ST2 contributes to carrageenin-induced innate inflammation, which is itself dependent on the activation of resident tissue cells and recruitment of leukocytes.

It is interesting to note that there is rapid release of IL-33 upon stimulation of endothelial and epithelial cells (Moussion *et al.*, 2008). This finding, together with its actions as a cytokine and an intracellular nuclear factor show similarities with the alarmin HMGB-1 and raise the possibility that IL-33 could be a novel alarmin (Carriere *et al.*, 2007; Moussion *et al.*, 2008; Byrne *et al.*, 2011). Compatible with a fast and early release of IL-33 in innate inflammation, this cytokine was produced in significant amounts upon carrageenin stimulus within 30 min, and production of IL-33 continued at significant levels, at least, up to 5 h. There was also induction of IL-33 and ST2 mRNA expression, which indicated a continuous stimulation of IL-33 expression and not only its release and/or activation. Similar levels of IL-33 and/or ST2 mRNA (Savinko *et al.*, 2012; Schmieder *et al.*, 2012) and IL-33 production (Alves-Filho *et al.*, 2010) have been found in a range of conditions. This early production of IL-33 induced by carrageenin could account for the early participation of IL-33/ST2 in carrageenin-induced oedema, at 30 min. In agreement with IL-33 participation in oedema, this cytokine increased endothelial permeability in an *in vitro* model by increasing NO levels (Choi *et al.*, 2009). Injection of IL-33 alone in WT mice mimicked the paw inflammatory effects of carrageenin. Although the oedema induced by IL-33 was statistically significant, it did not attain the magnitude of oedema after carrageenin. Carrageenin-induced oedema is known to involve several other mediators, which potentiate the activity of each other (Ferreira and Vargaftig, 1974; Williams *et al.*, 1983; Katz *et al.*, 1984). Thus, it is to be expected that IL-33 alone would not fully mimic the carrageenin-induced oedema. Furthermore, the doses of IL-33 used were chosen from a dose–response curve and were similar to previous data on induction of pain (Verri *et al.*, 2008) and to the doses of other single cytokines necessary to induce inflammation (Joosten *et al.*, 2006). For instance, IL-32γ, TNF-α and IL-1β induce significant joint oedema at 100 ng·per joint, as determined by ^{99m}Tc-uptake (Joosten *et al.*, 2006).

The participation of IL-33/ST2 in carrageenin-induced hyperalgesia was evident starting at 1 h, and IL-33 injection achieved similar levels of mechanical hyperalgesia compared with carrageenin. The inhibition of leukocyte recruitment by fucoidin suggests the participation of recruited leukocytes in the IL–33-induced mechanical hyperalgesia. Nevertheless, the role of recruited leukocytes in the IL–33-induced hyperalgesic response was limited because abolishing the recruitment of leukocytes only partially reduced the mechanical hyperalgesia and correlations between IL–33-induced hyperalgesia and myeloperoxidase activity were not evident at any of the doses tested. The ST2$^{-/-}$ mice also presented partial reduction of myeloperoxidase activity upon carrageenin stimulus. Furthermore, it is possible that the participation of IL-33/ST2 in the recruitment of leukocytes such as neutrophils would become more evident at later stages of inflammation because cytokines such as TNF-α induce the expression of ST2 in neutrophils (Verri *et al.*, 2010) and because IL-33 is processed into mature form by neutrophil elastase and cathepsin G (Lefrançais *et al.*, 2012) indicating that, as the inflammatory process continued, the cellular responsiveness to IL-33 would increase and the recruited neutrophils would further contribute to activation of IL-33.

There is activation of a cascade of cytokines in the carrageenin-induced mechanical hyperalgesia model in which TNF-α and CXCL1 induce the production of IL-1β that, in turn, induces the production of PGE$_2$ (Ferreira *et al.*, 1988; Cunha *et al.*, 1992; 2005). Furthermore, TNF-α- and IL–1β-induced mechanical hyperalgesia were inhibited by ET receptor antagonists (Verri *et al.*, 2006). Both ET$_A$ and ET$_B$ receptor antagonists reduce carrageenin-induced mechanical hyperalgesia (Baamonde *et al.*, 2004) and ET-1 induces PGE$_2$ production in immunized mice (Verri *et al.*, 2007). Thus, cytokines seem responsible for ET-1 production in carrageenin inflammation. In the present study, IL–33-induced mechanical hyperalgesia was inhibited by targeting TNF-α, CXCL1, IL-1β, ET$_A$ and ET$_B$ receptors, and PGE$_2$. Further, the carrageenin-induced production of TNF-α, CXCL1, IL-1β and PGE$_2$, and preproET-1 mRNA expression were inhibited in ST2$^{-/-}$ mice. Therefore, IL-33/ST2 signalling seems to be an early event in carrageenin-induced inflammatory mechanical hyperalgesia by inducing the production of cytokines, chemokines, ET-1 and PGE$_2$. In addition to the contribution of TNF-α, CXCL1, IL-1β, ET-1 and PGE$_2$ to inflammatory hyperalgesia, these mediators also contribute to oedema and/or leukocyte recruitment (Williams, 1982; Faccioli *et al.*, 1990;

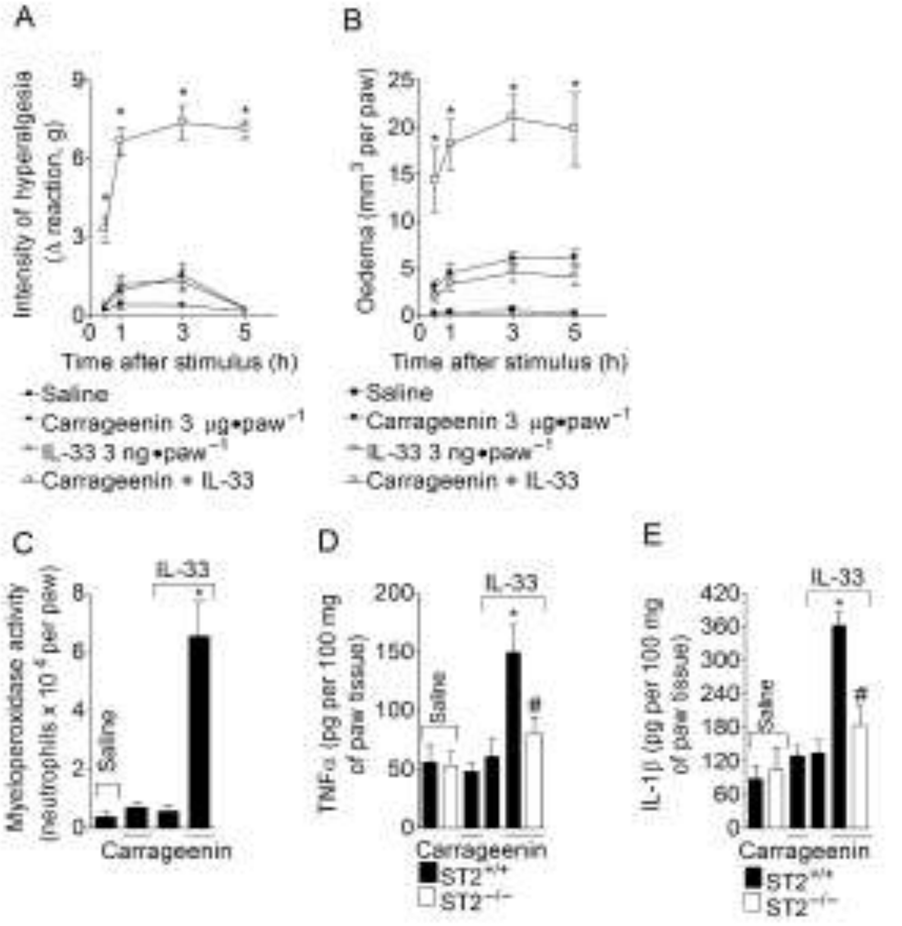

Figure 10

IL-33 synergizes with carrageenin to induce inflammation, hyperalgesia and cytokine production. Mice received i.pl. injection of IL-33 (3 ng-per paw), carrageenin (3 μg-per paw) or IL-33 plus carrageenin (same doses). Mechanical hyperalgesia (panel A) and oedema (panel B) were determined at indicated time points followed by myeloperoxidase activity determination at 5 h (panel C) in paw skin samples. TNF-α (panel D) and IL-1β (panel E) levels were determined by ELISA. *n* = 6 for hyperalgesia, oedema and myeloperoxidase activity, n = 4 for ELISA, representative of two separate experiments. *P < 0.05 compared with the saline, carrageenin or IL-33 in ST2$^{+/+}$ group and #P < 0.05 compared with the carrageenin plus IL-33 in ST2$^{+/+}$ mice group.

McColl and Clark-Lewis, 1999; Verri *et al.*, 2007; Joosten *et al.*, 2006; Conte *et al.*, 2008; Zarpelon *et al.*, 2012).

Treatment with soluble ST2-Fc fusion protein inhibits intestinal ischaemia/reperfusion-induced lethality and inflammation by inducing IL-10 production (Fagundes *et al.*, 2007). IL-10 is an endogenous anti-hyperalgesic cytokine in the carrageenin model (Poole *et al.*, 1995), and carrageenin induced production of IL-10 in our experiments. However, in ST2$^{-/-}$ mice, the output of this cytokine was reduced at 3 and 5 h, demonstrating clearly that the loss of hyperalgesia in the ST2$^{-/-}$ mice could not be due to increased IL-10 levels.

It is interesting to point out that IL-33 potentiates antigen-induced production of cytokines in a mast cell line (Andrade *et al.*, 2011). We therefore proposed that IL-33 could also exhibit such activity during carrageenin inflammation. We observed a synergy between IL-33 and carrageenin at doses that were ineffective, as single treatments, in inducing hyperalgesia, oedema and myeloperoxidase activity. Moreover, this synergy was also observed in terms of TNF-α and IL-1β production, and the synergy was ST2-dependent. Therefore, it is likely that, although relatively high doses of IL-33 given alone did induce inflammation and pain in normal mice, during carrageenin paw inflammation much lower doses of IL-33 are required because this cytokine synergizes with carrageenin.

In conclusion, we have shown here that IL-33/ST2 was involved in carrageenin-induced inflammatory oedema, leukocyte recruitment and mechanical hyperalgesia. The mechanisms triggered by IL-33/ST2 involve the production of pro-inflammatory cytokines, ET-1 and PGE_2. This prominent role of IL-33/ST2 suggests that it is now important to determine whether drugs that inhibit carrageenin-induced inflammation also affect IL-33/ST2 signalling and supports further pre-clinical and clinical studies on IL-33/ST2 targeting therapies in innate inflammation. Moreover, even if the primary use of therapies targeting IL-33/ST2 signalling is not intended to reduce pre-existing pain, they might contribute to reduce it. Pain is a likely side effect of therapies using recombinant IL-33 as it has been observed with other cytokines such as IL-12 and granulocyte-colony stimulating factor (Verri *et al*., 2005; Carvalho *et al*., 2011) although a non-steroidal anti-inflammatory drug would be sufficient to reduce such side effects, as suggested here.

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Conflict of interest

The authors declare no conflict of interest.

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4.2. Anexo 2

Interleukin-33/ST2 signaling mediates chronic constriction injury-induced neuropathic pain in mice: Participation of spinal PI_3K, mTOR, MAP kinases, NFκB, and glia.

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Running title: ST2 deficient mice and neuropathic pain.

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Abstract

Interleukin (IL)-33 is a member of IL-1 family of cytokines with pleiotropic functions. Herein, it was investigated the role of IL-33 in chronic constriction injury of the sciatic nerve (CCI)-induced neuropathic pain in mice. $ST2^{-/-}$ mice presented reduced mechanical hyperalgesia compared to background mice and CCI induced significant spinal production of IL-33, 7 days after surgery. The intratechal (i.t.) injection of IL-33 induced dose-dependent mechanical hyperalgesia, which was inhibited in $ST2^{-/-}$ and $TNFR1^{-/-}$ mice and animals treated with IL-1ra. The IL-33 i.t. injection also induced the production of TNFα and IL-1β in spinal cord samples (L4-L6) and CCI-induced TNFα and IL-1β production was reduced in $ST2^{-/-}$ mice. The mechanical hyperalgesia caused by i.t. injection of TNFα and IL-1β was reduced in $ST2^{-/-}$ mice and CCI suggesting a crosstalk among these cytokines. IL-33 i.t. injection also induced mechanical hyperalgesia which was amenable by inhibition of PI_3K, mTOR, ERK, JNK, p38 and NFκB. Furthermore, CCI-induced spinal activation of astrocytes (GFAP) and microglia (Iba-1) was dependent on ST2 as well as IL-33-induced hyperalgesia was inhibited by glia inhibitors fluorocitrate and minocycline. Therefore, IL-33/ST2 signaling is an important hyperalgesic pathway in CCI-induced neuropathic mechanical hyperalgesia by activating spinal glial cells and molecular signaling pathways related to pain.

Introduction

Neuropathic pain results from an injury of peripheral nervous system or central nervous system caused by some diseases, such as multiple sclerosis, cancer, diabetes, herpes zoster or trauma of nerves (Dworkin et al., 2003). It affects approximately 6% of worldwide population (Ma et al., 2012) with symptoms ranging from parestesia, spontaneous pain, allodynia to hyperalgesia. The treatment of neuropathic pain is still a challenge since 40 – 60% of patients have some relief with treatment (Sindrup and Jensen, 1999).

The healthy peripheral nerves are surrounded by numerous cells (schwann cells, fibroblasts, endothelial cells, macrophages and mast cells) that regulate the responses to tissue and never injury (Moalem and Tracey, 2006; Myers et al., 2006). Nerve injury result in pain behavior accompanied by an increase of proinflammatory cytokines, contributing to axonal damage (Keswani et al., 2003). Cytokine production and action in neuropathic pain are related to the activation of phosphatidylinositol 3 kinase (PI3K), mammaliam target of rapamycin (mTOR), mitogen-activated protein (MAP) kinases and NFκB (Schmitz et al., 2005). Furthermore, in response to damage of peripheral nerves, spinal nerves and spinal cord, both microglia and astrocytes in spinal cord are activated and their inhibition diminishes nociceptive transmission (McMahon et al., 2005; Watkins and Maier, 2003).

Interleukin-33 (IL-33) or IL-1F11 is the latest member of IL-1 family of cytokines, which includes IL-1α, IL-1β, IL-18, IL-36, IL-37 and IL-1Ra (Schmitz et al., 2005; Dinarello et al., 2013). IL-33 requires the expression of both ST2 receptor and IL-1 receptor accessory protein to function as a classical cytokine (Liu et al., 2013). ST2 receptor exists in transmembrane and soluble forms, which function as signaling receptor and decoy receptor, respectively (Trajkovic et al., 2004).

IL-33/ST2 signaling has pleiotropic roles in diseases in such way that it can be considered a target as well as treatment depending on cytokine milleu (Liew et al., 2012).

Focusing in pain, we have demonstrated that IL-33/ST2 signaling is important in antigen challenge-induced cutaneous and articular mechanical hyperalgesia. IL-33/ST2 induces further production of nociceptive molecules such as TNFα, IL-1β, IFNγ, endothelin-1 and prostaglandin E_2 (Verri et al., 2008). In addition to adaptive immune responses, IL-33/ST2 signaling mediates the hyperalgesia in the carrageenin innate inflammation model (Zarpelon et al., 2013) and overt pain-like behavior induced by acetic acid, phenyl-p-benzoquinone and formalin (Han et al., 2013; Magro et al., 2013). IL-33/ST2 signaling is also important in bone cancer pain (Zhao et al., 2013). However, it has not been investigated the molecular signaling pathways and the role of glia in IL-33/ST2 function in neurophatic pain. In the present study we demonstrated that IL-33/ST2 signaling is important in chronic constriction injury-induced neuropathic pain by activating PI_3K/AKT, mTOR, MAPK and NFκB signalling pathways, and an autocrine IL-33/ST2 signaling in glial cells.

Material and Methods

Animals

The experiments were performed on male Balb/C, ST2 deficient ($^{-/-}$) Balb/C background, C57BL/6 and TNFR1$^{-/-}$ C57BL/6 background mice weighing between 20 and 25g. Mice were housed in temperature-controlled rooms (22-25C), with access to water and food *ad libitum*. All experiments were conducted in accordance with animals' care and handling procedures were accordance with the Internacional Association for Study of Pain (IASP) and with the approval of the Ethics Committee of the Faculty of Medicine of Ribeirao Preto (University of Sao Paulo) and Universidade Estadual de Londrina. The animals were used only in a single experimental group.

Drugs and stimuli

The following materials were obtained from the sources indicated. Wortmanin, rapamycin, PD98059, SP600125, SB202190, PDTC, minocycline and fluorocitrate were obtained from Sigma Aldrich (St Louis, MO, USA). Recombinant rat TNF-α and human IL-1β were acquired from the National Institute for Biological Standards and Control (South Mimms, Hertfordshire, UK). Recombinant Human IL-33 was produced as previously described (Komai-Koma et al., 2007).

Model of chronic constriction injury (CCI)

Mice were anesthetized with ketamine and xilazine (10µL/ 10g) followed by trichotomy in the surgery area. The incision was performed in the rear leg, and the sciatic nerve was exposed with glass rod. A moderate constriction injury was performed around the sciatic nerve with a chrome suture. For the sham-operate controls, mice underwent the same procedure without the constriction of nerve.

Electronic pressure-meter test

The mechanical hyperalgesia test (Cunha et al., 2004) consisted of evoking a hindpaw flexion reflex with a hand-held force transducer (electronic anaesthesiometer; IITC Life Science) adapted with a 0.5 mm^2 polypropylene tip. The end point was the removal of the paw followed by clear flinching movements. After the paw withdrawal the intensity of the pressure was automatically recorded, and the value for the response was obtained by averaging three measurements. The animals were tested before and after treatments. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the mean measurements 3 h after stimulus from the zero-time mean measurements. Withdrawal threshold was 8.6 ± 0.5 g (mean ± s.e.m., n=40) before injection of the hyperalgesic agents.

Cytokine measurement

At indicated times after CCI animals were terminally anaesthetized and the spinal cord L4-L6 samples were removed and homogenized in 300 µl of buffer containing protease inhibitors. TNFα, IL-1β (BD Bioscience) and IL-33 (eBioscience) concentrations were determined by ELISA using paired antibodies.

Western blotting assay

Mice were terminally anesthetized on isoflurane chamber and the L4-L6 segments of the spinal cord were dissected out immediately and homogenised in RIPPA buffer, containing protease and fosfatase inhibitor. Afterwards, the lysates were crushed and centrifuged (130 rcf, 10 min, 4^oC). The proteins were separated by SDS-PAGE gel, at 6% between 10%, and transferred on PVDF transfer membrane (for mTOR analysis) or nitrocellulose membrane for the other analysis (GE Healthcare-Amersham, Pittsburgh, PA, USA). After, membranes were incubated in blocking buffer (5% nonfat milk in tris-buffered saline with tween 20, TBS-T, or 1% BSA) for different times for each antibody at 4^{o}C and incubated overnight at 4ºC in the presence of primary antibody. The primary and secondary antibodies used were in blocking buffer. The antibodies used were: phospho and total p38 (sc7975, sc535); phospho and total JNK (#9251, #9252); phosphor and total ERK (#9101, #9102); phospho and total IκBα (#2859, sc371); phospho and total AKT (#9271, #9272; PI3 Kinase p110γ and β-actina (#4252, #4970); GFAP (#3670) and Iba-1 (WAKO - Cat.016-20.001), IL-33 and β-actina (sc98660, #4970). For evaluation of activity of mTOR were used 2 antibodies: p-mTOR (Ser 2448) (#2971) and p-mTOR (Ser 2481) (#2974) and secondary total mTOR (#2972) Protein weights were measured against Precision Plus protein standards (Bio-Rad, Hercules, CA, USA). After being washed in phosphate-buffered saline with Tween 20, the membrane was incubated with a secondary antibody for 2 h at room temperature. Proteins were visualized by chemiluminescence with ECL detection reagent (GE Healthcare-Amersham, Pittsburgh, PA, USA). The membranes were reprobed with antibody to β-actin or to the total protein of interest for use as loading control.

Experimental protocols

In the first series of experiments, CCI was induced in ST2$^{+/+}$ and ST2 deficient ($^{-/-}$). The intensity of mechanical hyperalgesia was measured 1-23 days by the electronic pressure-meter test. IL-33 expression was determined in spinal cord samples (L4-L6) by ELISA (7 days after CCI) and western blot (3, 7 and 14 days after CCI). Mice received IL-33 injection (10, 30 and 100 ng, i.t.) and the mechanical hyperalgesia was between 1 - 48h. The dose of 100 ng of IL-33 i.t. was used in all following experiments with injection of IL-33. To confirm whether IL-33 was acting on ST2, IL-33 was injected in ST2$^{-/-}$ mice. IL-33 was also injected in CCI ST2$^{+/+}$ and ST2$^{-/-}$ mice and the mechanical hyperalgesia was evaluated at 1 - 24h. ST2 deficient mice received i.t. injection of TNFα (1 ng) and IL-1β (1 ng) and the mechanical hyperalgesia was evaluated at 1 - 24h. TNFα- and IL-1β-induced IL-33 production were evaluated in the spinal cord 2h after injection. IL-33-induced hyperalgesia (1 – 24h) was also target using TNFR1$^{-/-}$ mice and IL-1ra treatment. IL-33-induced production of TNFα and IL-1β in the spinal cord as well as the effect of ST2$^{-/-}$ in CCI-induced production of TNFα and IL-1β were determined at 2h and 7 days, respectively. To determine the molecular mechanisms triggered by IL-33/ST2, mice were co-treated with IL-33 and one of the following inhibitors: wortmanin (PI_3K inhibitor; 1-10 µg), rapamycin (mTOR inhibitor; 1-10 µg), PD98059 (MEK1 inhibitor, prevents ERK activation; 1-10 µg), SP600125 (JNK inhibitor; 1-10 µg), SB202190 (p38 inhibitor; 1-10 µg) and PDTC (pyrrolidine dithiocarbamate, NFκB inhibitor, 30 and 300 µg). Three, 7, 14 and 21 days after CCI in ST2$^{+/+}$ and ST2$^{-/-}$ mice, it was determined by western blot whether ST2 deficiency affected the activation of those signaling pathways. Possible effect of IL-33/ST2 in glial cells was evaluated by co-injection (i.t.) of IL-33 (100 ng) and minocycline (microglia inhibitor; 15, 50 and 150 µg) or fluorocitrate (astrocytes inhibitor; 0.53, 1.6 and 4.8) followed by evaluation of mechanical hyperalgesia 1 - 7h. The effect of minocyclin and fluorocitrate on CCI-induced mechanical hyperalgesia was also determined on day 7. The ST2-dependent expression of Iba-1 and GFAP in CCI was evaluated by western blot.

Statistical analyses

Results are representative of two independent experiments and are presented as the means ± s.e.m. (n = 6 [Figs. 1-6, 9] or 4 [Figs. 7-8, 10] per group in each experiment). Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times when the parameters were measured at different times after the stimulus injection. The analyzed factors were treatments, time and time *versus* treatment interaction. One-way ANOVA followed by Tukey's t test was performed for each time. Comparison of two groups was performed using t test. $P<0.05$ was considered significant.

Results

ST2 deficiency ($^{-/-}$) inhibits Chronic Constriction Injury (CCI)-induced mechanical hyperalgesia

CCI induced significant mechanical hyperalgesia between 3-23 days (Fig. 1A). After 23 days, the mechanical hyperalgesia began to decline, but remained significant up to 35 days (data not shown). CCI-induced mechanical hyperalgesia was inhibited in ST2$^{-/-}$ mice (Fig. 1A). In agreement, CCI induced significant IL-33 production in the spinal cord (L4-L6) compared to sham 7 days after surgery as determined by ELISA (Fig. 1B) and at 3 and 7 days as determined by western blot (Fig. 1C). It was also observed in a different model of neurophatic pain, the spinal nerve ligation (SNL)-induced mechanical hyperalgesia was reduced in ST2$^{-/-}$ compared to ST2$^{+/+}$ mice (Supplementary Figure S1).

Intratechal injection of IL-33 induces and increases CCI mechanical hyperalgesia in a ST2-dependent manner

The i.t. injection of IL-33 induced significant mechanical hyperalgesia with the three doses tested (10, 30 and 100 ng/mice). Nevertheless, 100 ng of IL-33 reached the highest response and with significant differences compared to the other doses (Fig. 2A). Therefore, the dose of 100 ng of IL-33 was chosen for next experiments. The mechanical hyperalgesia induced by i.t. injection of IL-33 was abolished in ST2$^{-/-}$ mice (Fig. 2B). Furthermore, i.t. injection of IL-33 increased the mechanical hyperalgesia in CCI mice in a ST2-dependent manner (Fig. 3C).

TNFα and IL-1β induce IL-33/ST2-dependent mechanical hyperalgesia

The i.t. injection of TNFα (Fig. 3A) and IL-1β (Fig. 3B) induced significant mechanical hyperalgesia, which was abolished in ST2$^{-/-}$ mice. Moreover, the i.t. injection of TNFα (Fig. 3C) and IL-1β (Fig. 3D) increased the production of IL-33 in the spinal cord (L4-L6).

IL-33 induces TNFα- and IL-1β-dependent mechanical hyperalgesia

The mechanical hyperalgesia induced by the i.t. injection of IL-33 was reduced in TNFR1$^{-/-}$ (Fig. 4A) and IL-1ra treated (Fig. 4B) mice. Furthermore, IL-33 i.t. injection increased the spinal levels of TNFα (Fig. 4C) and IL-1β (Fig. 4D). The CCI-induced production of TNFα (Fig. 4E) and IL-1β (Fig. 4F) was attenuated in ST2$^{-/-}$ mice 7 (Fig. 4E and 4F) and 14 (data not shown) days after surgery. Results of Figs. 3 and 4 indicate a cross-talk between IL-33, TNFα and IL-1β.

The i.t. injection of IL-33 induces mechanical hyperalgesia dependent on spinal activation of PI$_3$K, mTOR, MAP kinases and NFκB signaling in mice

Mice received i.t. co-injection of IL-33 (100 ng) and one of the following inhibitors: wortmanin (Fig. 5A, PI3K inhibitor), rapamycin (Fig. 5B, mTOR inhibitor), PD98059 (Fig. 5C, MEK1/2 inhibitor, prevents ERK1/2 activation), SB202190 (Fig. 5D, p38 inhibitor) or SP600125 (Fig. 5E, JNK inhibitor) at doses of 1, 3 and 10 µg/mice, or PDTC (Fig. 5F, NFκB inhibitor) at doses of 30 and 300 µg/mice. The dose of 10 µg/mice of wortmanin, rapamycin, PD98059, SB202190 and SP600125, or the dose of 300 µg/mice of PDTC reached the highest inhibitory effect over IL-33-induced mechanical hyperalgesia, and with significant differences compared with the other doses. Therefore, IL-33 hyperalgesia depends on spinal activation of PI$_3$K, mTOR p38, ERK, JNK, and NFκB. The dose of 10 µg/mice for all inhibitors except PDTC (300 µg/mice) were selected.

CCI-induced mechanical hyperalgesia depends on spinal activation of PI_3K, mTOR, MAP kinases and NFκB signaling in mice

At 7 (Fig. 6) and 14 days (data not show) after CCI mice received an i.t. treatment with wortmanin (Fig. 6A, PI3K inhibitor), rapamycin (Fig. 6B, mTOR inhibitor), PD98059 (Fig. 6C, MEK1/2 inhibitor, prevents ERK1/2 activation), SB202190 (Fig. 6D, p38 inhibitor) and SP600125 (Fig. 5E, JNK inhibitor) at 10 µg/mice, and PDTC at 300 µg/mice (Fig. 6F). All inhibitors reduced ongoing CCI-induced mechanical hyperalgesia in mice. Therefore, the mechanisms triggered by i.t. injection of IL-33 are relevant in CCI model and amenable by i.t. treatment similarly to IL-33.

IL-33/ST2 is required for activation of PI_3K, AKT and mTOR signaling in the spinal cord of CCI mice

CCI induced significant mechanical hyperalgesia between 3-23 days show on Fig. 1A, therefore, samples for determination of PI_3K, AKT and mTOR were collected at days 3, 7, 14 and 21 days after CCI induction. Western blot for PI_3K and β-actin, and IOD ratio (Fig. 7A), phospho-AKT (Ser 473) and total AKT, and IOD ratio (Fig. 7B), phospho-mTOR (which is phosphorylated at Ser2448 via PI3 kinase/Akt signaling pathway) and total mTOR, and IOD ratio (Fig. 7C), phospho-mTOR (autophosphorylated at Ser2481) and total mTOR, and IOD ratio (Fig. 7D) were performed. The results showed that CCI induced an increase of phosphorylation of PI_3K (at days 3 and 7) (Fig. 7A), AKT at day 3 (Fig. 7B), mTOR on residue Ser2448 at days 7, 14 and 21 (Fig. 7C) and mTOR on autophosphorylated residue at day 3 (Fig. 7D). The effect of CCI on protein phosphorilation was reduced on $ST2^{-/-}$ mice in the same time points. Therefore, the PI_3K/ AKT/ mTOR pathway is relevant in CCI-induced hyperalgesia, and dependent on IL-33/ST2 signaling to be activated in the spinal cord (Salmond et al., 2012).

CCI-induced p38, ERK1/2, JNK and NFκB activation depends on IL-33/ST2 signaling in mice

Western blot was performed in samples collected at days 3, 7, 14 and 21 after CCI surgery in $ST2^{+/+}$ and $ST2^{-/-}$ mice. Representative western blotting of phosphor-p38, total p38 and IOD ratio (Fig. 8A), phosphor-ERK 1/2, total ERK 1/2 and IOD ratio (Fig. 8B), phosphor-JNK, total JNK and IOD ratio (Fig. 8C), phospho-IκBα (ser32), total IκBα and IOD ratio (Fig. 8D) are shown as indicated. CCI induced the phosphorylation of p38 at days 3 and 7 (Fig. 8A), ERK1/2 at day 7 (Fig. 8B) and JNK at days 3 and 7 (Fig. 8C), and degradation of IκB at days 7 and 14 (Fig. 8D). The CCI-induced activation of p38, ERK1/2, JNK and IκB/NFκB was reduced in $ST2^{-/-}$ compared to $ST2^{+/+}$ mice (Fig. 8). Therefore, CCI-induced spinal activation of p38, ERK1/2, JNK and NFκB depends on IL-33/ST2 signaling.

Autocrine glia activation depends on IL-33/ST2 signaling in CCI-induced hyperalgesia

The i.t. treatment with minocycline (15, 50 and 150 µg/mice, microglia inhibitor (Lu, 2013) (Fig. 9A) and fluorocitrate (0.53, 1.6 and 4.8 µg/mice, astrocytes inhibitor (Liu et al., 2012) (Fig. 9B) reduced the mechanical hyperalgesia induced by i.t. injection of IL-33 (100 ng) in mice. The doses of 50 µg/mice of minocycline and 1.6 µg/mice of fluorocitrate reached the maximal inhibition when compared with the other doses tested. In the CCI model, the treatment with minocycline (Fig. 9C) and fluorocitrate (Fig. 9D) at day 7 after surgery reduced ongoing mechanical hyperalgesia in mice. Furthermore, minocycline and fluorocitrate also reduced CCI-induced IL-33 levels in the spinal cord 3 h after treatment as determined by ELISA (Fig. 9E). Therefore, microglia and astrocytes are activated by IL-33 in the spinal cord as well as these cells participate in CCI-induced mechanical hyperalgesia and IL-33 production.

The IL-33/ ST2 signaling are essential to glial cells activation in neuropathic pain

Western blot was performed in spinal cord (L4-L6) samples collected 3, 7, 14 and 21 days after CCI surgery in ST2$^{+/+}$ and ST2$^{-/-}$ mice. To determine the participation of glial cells in the CCI-induced neuropathic pain, western blot was performed to determined the expression of Iba-1 to evaluated the activation of microglia (Fig. 10A), and GFAP to determine activation of astrocytes (Fig. 10B) using β-actin as control. Representative western blot of Iba-1 (Fig. 10A) shows an increase of Iba-1 expression at days 3 and 7 in a ST2-dependent manner. GFAP expression was increased at days 7 and 14 in a ST2-dependent manner. Therefore, IL-33/ST2 signaling participates in CCI-induced spinal cord activation of microglia and astrocytes.

Conclusion and Discussion

IL-33 is an intriguing molecule due to its pleiotropic roles. Despite the evidence of its participation in inflammatory and cancer pain models, the role of IL-33/ST2 signaling in neuropathic pain and molecular signaling nociceptive pathways remained to be determined. In the present study, it was demonstrated that i.t. injection of IL-33 induces mechanical hyperalgesia via ST2 and dependent on cytokines (TNFα and IL-1β), PI_3K, mTOR, MAPK and NFκB, and glial cells. Moreover, CCI-induced mechanical hyperalgesia was dependent on the same signaling pathways that were triggered in the spinal cord of CCI mice in a ST2-dependent manner. Furthermore, there is an autocrine regulation of microglia and astrocytes by IL-33/ST2 in CCI.

IL-33/ST2 signaling mediates the cutaneous and articular mechanical hyperalgesia induced by antigen challenge in mice by triggering the production of TNFα, IL-1β, IFNγ, ET-1 and PGE_2 (Verri et al., 2008). In the carrageenin model, it was observed that IL-33/ST2 mediates the mechanical hyperalgesia, paw edema and cellular recruitment by inducing the production of TNFα, CXCL1, IL-1β, ET-1 and PGE_2. IL-33 also potentiated the hyperalgesic effect of carrageenin (Zarpelon et al., 2013). In a model of bone cancer pain induced by injection of 4T1 mammary carcinoma cells, the i.t. treatment with ST2 antibody or injection in $ST2^{-/-}$ mice reduced the mechanical and thermal hyperalgesia. Co-expression of IL-33 and GFAP was observed at basal conditions and the increase of spinal IL-1β, IL-6 and TNFα in the bone cancer $ST2^{+/+}$ group did not occur in ST2-/- mice (Zhao et al., 2013). In the formalin model, ST2 deficiency or anti-ST2 antibody reduces the nociceptive responses (Han et al., 2013; Magro et al., 2013) and i.t. injection of IL-33 induces paw lifting and licking (Han et al., 2013). Therefore, the present knowledge on IL-33/ST2 role in pain is in agreement with our first finding indicating a role for IL-33/ST2 in the further production of nociceptive molecules (Verri et al., 2008). In the present study, it was also observed that i.t. injection of

TNFα and IL-1β induce IL-33 production and mechanical hyperalgesia dependent on IL-33/ST2 as well as i.t. injection of IL-33 mediates TNFα and IL-1β in the spinal cord and induces hyperalgesia dependent on TNFα and IL-1β in the CCI model. IL-33 i.t. injection also increased CCI hyperalgesia. Importantly, CCI- and SNL-induced mechanical hyperalgesia was reduced in $ST2^{-/-}$ compared to $ST2^{+/+}$ mice. Therefore, although our study focused in the CCI model, the role of IL-33/ST2 signaling might be wider and relevant in other neuropathic pain condition.

It is important to advance in the intracellular molecular mechanisms triggered by IL-33/ST2. In experimental settings not related to pain, it was observed in vitro that IL-33/ST2 triggers the activation of MAP kinases and NFκB (Schmitz et al., 2005), and one study detected the activation of PI3K and mTOR by IL-33 (Ivanov et al., 2010). In neuropathic pain models, there is evidence of the participation of PI_3K, mTOR, MAP kinases and NFκB. In the CCI model, it was observed the participation of mTOR (Zhang et al., 2013), p38 (Kim et al., 2002), ERK (Lim et al., 2003), JNK (Di Cesare Mannelli et al., 2010) and NFκB (Chu et al., 2012). Therefore, we reason that IL-33/ST2 role in CCI-induced neuropathic pain could also involve the signaling pathways previously described for IL-33/ST2.

The mechanical hyperalgesia induced by i.t. injection of IL-33 was diminished in a dose-dependent manner by inhibitors of PI_3K, mTOR, MAP kinases (p38, ERK1/2 and JNK) and NFκB. In the CCI model, the same inhibitors reduced the hyperalgesia 7 days after surgery, indicating that CCI-induced hyperalgesia depends on the same signaling pathways triggered by IL-33/ST2. Furthermore, CCI-induced increased phosphorilation of PI_3K, AKT, mTOR, MAP kinases and NFκB, which was reduced in $ST2^{-/-}$ mice. Therefore, IL-33/ST2 signaling contributes to CCI-induced mechanical hyperalgesia by triggering the activation of PI_3K, mTOR, MAP kinases (p38, ERK1/2 and JNK) and NFκB. These data is in accordance with the reduced production of TNFα and IL-1β in CCI $ST2^{-/-}$ compared to $ST2^{+/+}$ mice. Of note, to our knowledge, this is the first evidence that PI_3K have a role in CCI-induced neuropathic pain.

The time profile of western blot analysis of CCI neuropathic pain reveled that most of the molecular mechanisms addressed were activated at days 3 and 7 except for NFκB (IκB degradation) that initiated at day 7 peaking at day 14. mTOR activation was the only molecule with sustained activation at day 21, which is consistent with the activation of lymphocytes by IL-33 (Salmond et al., 2012) and the involvement of these cells latter on neuropathic pain conditions.

IL-33 is produced by endothelial cells and astrocytes but not by microglia or neurons in basal conditions (Yasuoka et al., 2011). This is in agreement with the observation of co-localization of IL-33 and GFAP (Zhao et al., 2013). ST2 and IL-1RAcP are expressed mainly in microglia and astrocytes, which corroborates that IL-33 dose-dependently induces the proliferation of microglia and enhances the production of pro-inflammatory cytokines TNFα and IL-1β, and the anti-inflammatory cytokine IL-10 (Yasuoka et al., 2011). These data are in accordance with the present results demonstrating that the hyperalgesia induced by i.t. injection of IL-33 depends on the activation of microglia and astrocytes and that IL-33/ST2 signaling is important for the activation of microglia and astrocytes during CCI neuropathic pain condition. It is possible that IL-33 could activate astrocytes and endothelial cells to produce TNFα and IL-1β, which in turn activate microglia and neurons. These cellular communications mediated by IL-33/ST2 signaling may also depend on disease context. In a model of autoimmune encephalomyelitis, IL-33 was co-expressed with NeuN and GFAP, indicating the IL-33 presence in neurons and astrocytes, respectively. ST2 was co-expressed with NeuN, indicating its expression in neurons (Jiang et al., 2012). Therefore, in the autoimmune encephalomyelitis model, IL-33 could be produced by neurons and astrocytes and activate neurons.

Concluding, IL-33/ST2 signaling is important in CCI- and SNL-induced neuropathic pain. IL-33/ST2 mediates CCI neuropathic pain by triggering the production of cytokines TNFα and IL-1β as well as activating intracellular signaling pathways named PI_3K, mTOR, MAP kinases (p38, ERK and JNK) and NFκB with activation of microglia and astrocytes. Therefore, the prominent role of IL-33/ST2 signaling in neuropathic pain and other pain conditions, suggest it as a promising analgesic target to be further investigated.

Acknowledgments

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Disclosure

The authors declare no conflict of interest.

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Figure legends

Fig. 1. IL-33/ST2 mediates chronic constriction injury (CCI)-induced hyperalgesia. CCI or sham operation was induced in ST2$^{+/+}$ and ST2$^{-/-}$ mice. Mechanical hyperalgesia was evaluated at indicated time points (panel A). IL-33 levels were determined 7 days after CCI induction by ELISA (Panel B) and 3 and 7 days by western blot (Panel C). n = 6, representative of 2 separated experiments. $*P < 0.05$ compared to sham group and $\#P < 0.05$ compared to CCI ST2$^{+/+}$ group.

Fig. 2: Intratechal (i.t.) injection of IL-33 induces ST2-dependent hyperalgesia in naïve mice and CCI mice. IL-33 (10, 30 and 100 ng) was injected i.t. in ST2$^{+/+}$ naive mice (Panel A), IL-33 (100 ng) was injected in naïve ST2$^{+/+}$ and ST2$^{-/-}$ mice (Panel B). IL-33 (100 ng) was injected in ST2$^{+/+}$ and ST2$^{-/-}$-induced CCI mice (Panel C). Mechanical hyperalgesia was evaluated at 1-48h in naïve mice, and at 7 days after CCI induction IL-33 was injected and mechanical hyperalgesia determined between 1-24h. n = 6, representative of 2 separated experiments. $*P < 0.05$ compared to saline or sham group, $\#P < 0.05$ compared to IL-33 at 10ng, IL-33 in ST2$^{+/+}$ mice or vehicle ST2$^{+/+}$ CCI group.

Fig. 3: TNFα and IL-1β induce IL-33/ST2-dependent mechanical hyperalgesia. ST2$^{+/+}$ and ST2$^{-/-}$ received 1 ng of TNFα (Panel A) or IL-1β (Panel B) i.t., and mechanical hyperalgesia was evaluated 1-24 h after. IL-33 levels in the spinal cord (L4-L6) were determined 2 h after i.t. injection of 1 ng of TNFα (Panel C) or IL-1β (Panel D) by ELISA. n = 6, representative of 2 separated experiments. $*P < 0.05$ compared to saline group and $\#P < 0.05$ compared to TNFα ST2$^{+/+}$ or IL-β ST2$^{+/+}$ group.

Fig. 4: IL-33 induces TNFα- and IL-1β-dependent mechanical hyperalgesia. IL-33 was injected (100 ng, i.t.) in naïve TNFR1$^{-/-}$ (Panel A) or IL-1ra treated (Panel B) mice and the mechanical hyperalgesia was evaluated 1-7h after. Two h after IL-33 i.t. injection, spinal cord samples were collected for TNFα (Painel C) and IL-1β (Panel D) measurement by ELISA. Seven days after CCI, TNFα (Panel E) and IL-1β (Panel F) levels were determined by ELISA in ST2$^{+/+}$ and ST2$^{-/-}$ mice. n = 6, representative of 2 separated experiments. *$P < 0.05$ compared to saline or sham group and #$P < 0.05$ compared to IL-33 TNFR1$^{+/+}$, IL-33 vehicle of IL-1ra or CCI ST2$^{+/+}$ group.

Fig. 5: The i.t. injection of IL-33 induces mechanical hyperalgesia dependent on spinal activation of PI3K, MAP kinases, NFκB and mTOR in mice. Mice received co-injection of IL-33 (100 ng) and one of the following drugs: wortmanin (PI3K inhibitor; 1-10 µg) (Panel A), rapamycin (mTOR inhibitor; 1-10 µg) (Panel B), PD98059 (MEK1 inhibitor, prevents ERK activation; 1-10 µg) (Panel C), SP600125 (JNK inhibitor; 1-10 µg) (Panel D), SB202190 (p38 inhibitor; 1-10 µg) (Panel E) and PDTC (pyrrolidine dithiocarbamate, NFκB inhibitor, 30 and 300 µg) (Panel F). Mechanical hyperalgesia was evaluated at indicated time points after IL-33 injection. n = 6, representative of 2 separated experiments. *P<0.05 compared saline group. #P<0.05 compared to vehicle + IL-33 group. **P<0.05 compared to the lower dose of drug tested. One-way ANOVA followed by Tukey's t test.

Fig. 6: Participation of PI3K, MAP kinases, NFκB and mTOR in CCI-induced mechanical hyperalgesia. The mechanical hyperalgesia of CCI mice was evaluated at the 6th to 8th days after surgery. In the 7th day, mice were treated with one of the following drugs: wortmanin (PI3K inhibitor; 10 µg) (Panel A), rapamycin (mTOR inhibitor, 10 µg) (Panel B), PD98059 (MEK1 inhibitor; 10 µg) (Panel C), SP600125 (JNK inhibitor; 10 µg) (Panel D),

SB202190 (p38 inhibitor; 10 μg) (Panel E) and PDTC (NFκB inhibitor; 300 μg) (Panel F). Mechanical hyperalgesia was evaluated at indicated time points after treatment. n = 6, representative of 2 separated experiments. *P<0.05 compared to CCI control group. One-way ANOVA followed by Tukey's t test.

Fig. 7. Spinal activation of AKT, PI3K and mTOR during CCI. CCI or sham operation was induced in $ST2^{+/+}$ and $ST2^{-/-}$ mice and representative Western blot was realized 3, 7, 14 and 21 days after surgery. Representative Western blots of PI3 kinase p110γ and β-actin (Panel A), Phospho AKT (Ser 473), total AKT and quantification of relative levels of AKT (Panel B), phospho mTOR (phosphorylated at Ser2448 via PI3 kinase/ Akt signaling pathway) and total mTOR (Panel C), phospho mTOR (autophosphorylated at Ser2481) and total mTOR (Panel D). n = 4, representative of 2 separated experiments. *$P < 0.05$ compared to sham group and #$P < 0.05$ compared to CCI $ST2^{+/+}$ group.

Fig. 8. Spinal activation of MAP kinases and NFκB during CCI. CCI or sham operation was induced in $ST2^{+/+}$ and $ST2^{-/-}$ mice and representative Western blot was realized 3, 7, 14 and 21 days after surgery. Representative Western blots of phospho p38, total p38 and quantification of relative levels of p38 (Panel A), phospho ERK 1/2 and total ERK 1/2 (Panel B), phospho JNK and total JNK (Panel C), Phospho IκBα (ser32) and total IκBα (Panel D). n = 4, representative of 2 separated experiments. *$P < 0.05$ compared to sham group and #$P < 0.05$ compared to CCI $ST2^{+/+}$ group.

Fig. 9: The i.t. injection of IL-33 and CCI-induces mechanical hyperalgesia dependent of microglia and astrocytes in mice. Mice received co-injection (i.t.) of IL-33 (100 ng) and minocycline (microglia inhibitor; 15, 50 and 150 μg) (Panel A) or fluorocitrate (astrocytes

inhibitor; 0.53, 1.6 and 4.8) (Panel B). Mechanical hyperalgesia was evaluated at indicated time points after IL-33 injection. The mechanical hyperalgesia of CCI mice was evaluated at the 6th to 8th days after surgery. In the 7th day, mice were treated with minocycline (Panel C) and fluorocitrate (Panel D), and the spinal cord was collected for IL-33 level analysis (Panel E). n = 6, representative of 2 separated experiments.*P<0.05 compared saline group. #P<0.05 compared to vehicle + IL-33 group. **P<0.05 compared to the lower dose of drug tested. One-way ANOVA followed by Tukey's t test.

Fig. 10. Microglia and astrocytes are important cells during CCI. CCI or sham operation was induced in $ST2^{+/+}$ and $ST2^{-/-}$ mice and representative Western blot was realized 3, 7, 14 and 21 days after surgery. Representative Western blots of Iba-1 and β-actin (Panel A) and GFAP and β-actin (Panel B). Quantification of relative levels of Iba-1 (Panel A), GFAP (Panel B). n = 4, representative of 2 separated experiments. *$P < 0.05$ compared to sham group and #$P < 0.05$ compared to CCI $ST2^{+/+}$ group.

Figure 1.

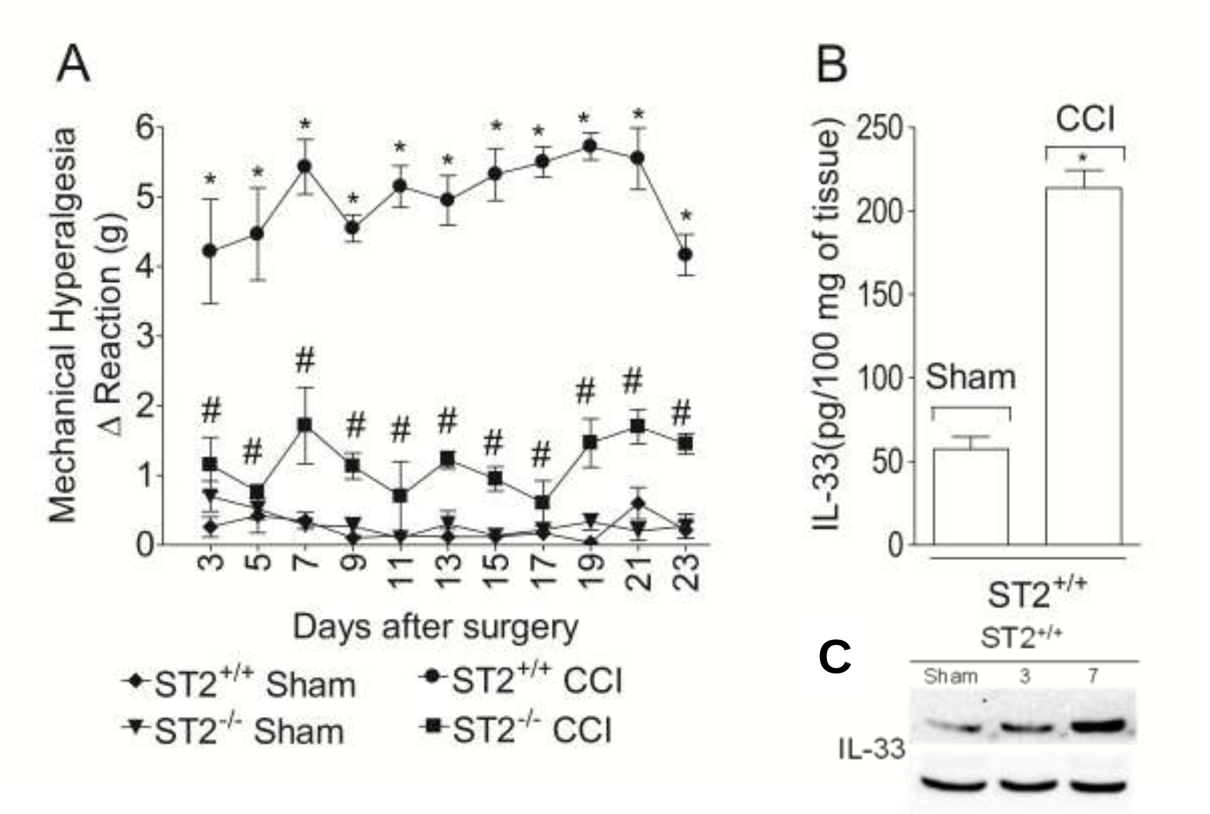

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Figure 2.

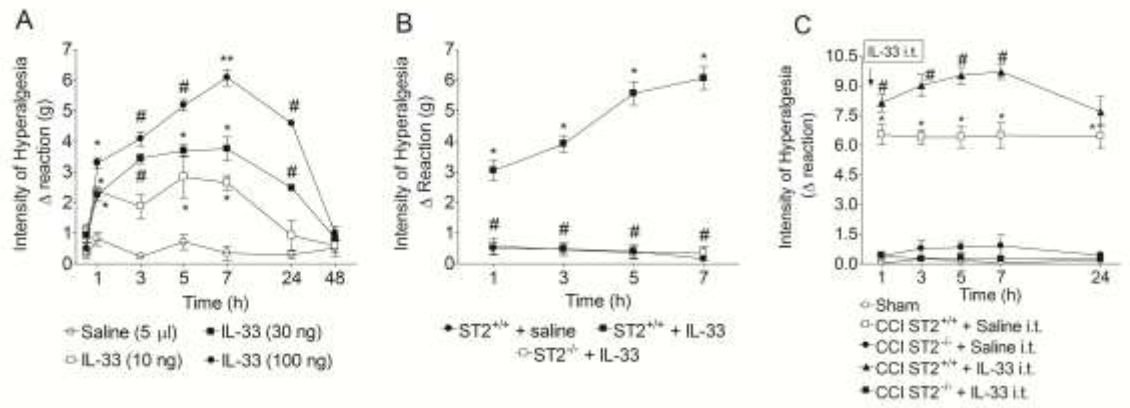

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Figure 3.

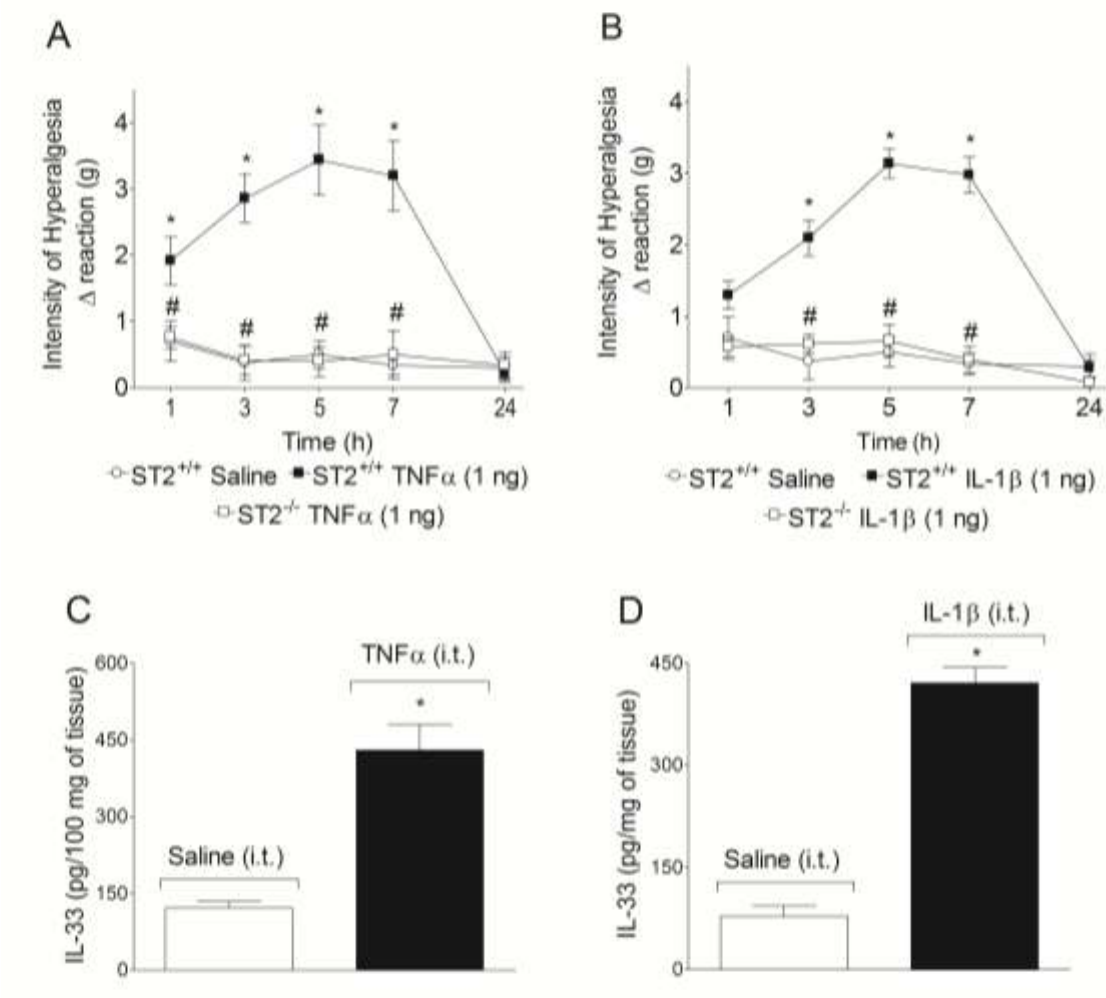

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Figure 4

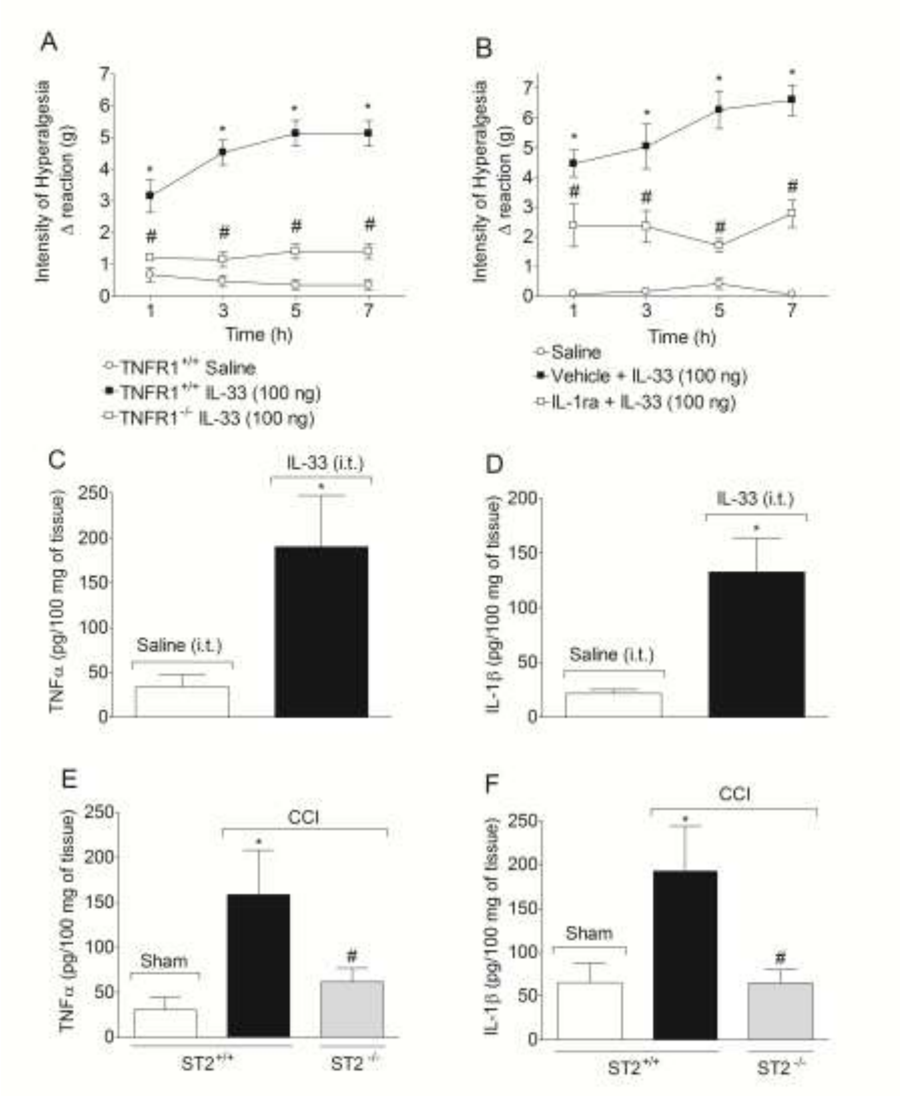

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Figure 5

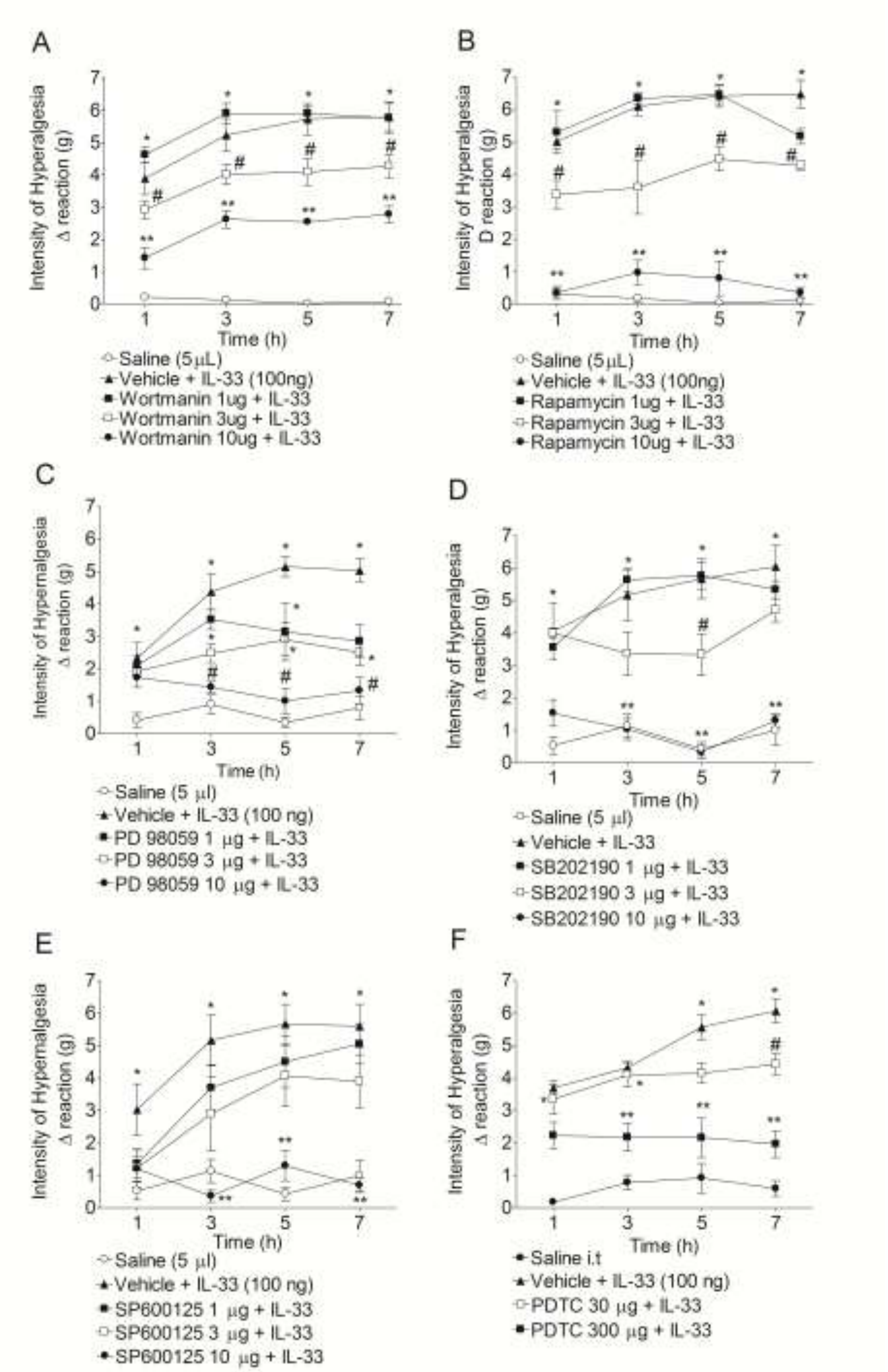

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Figure 6

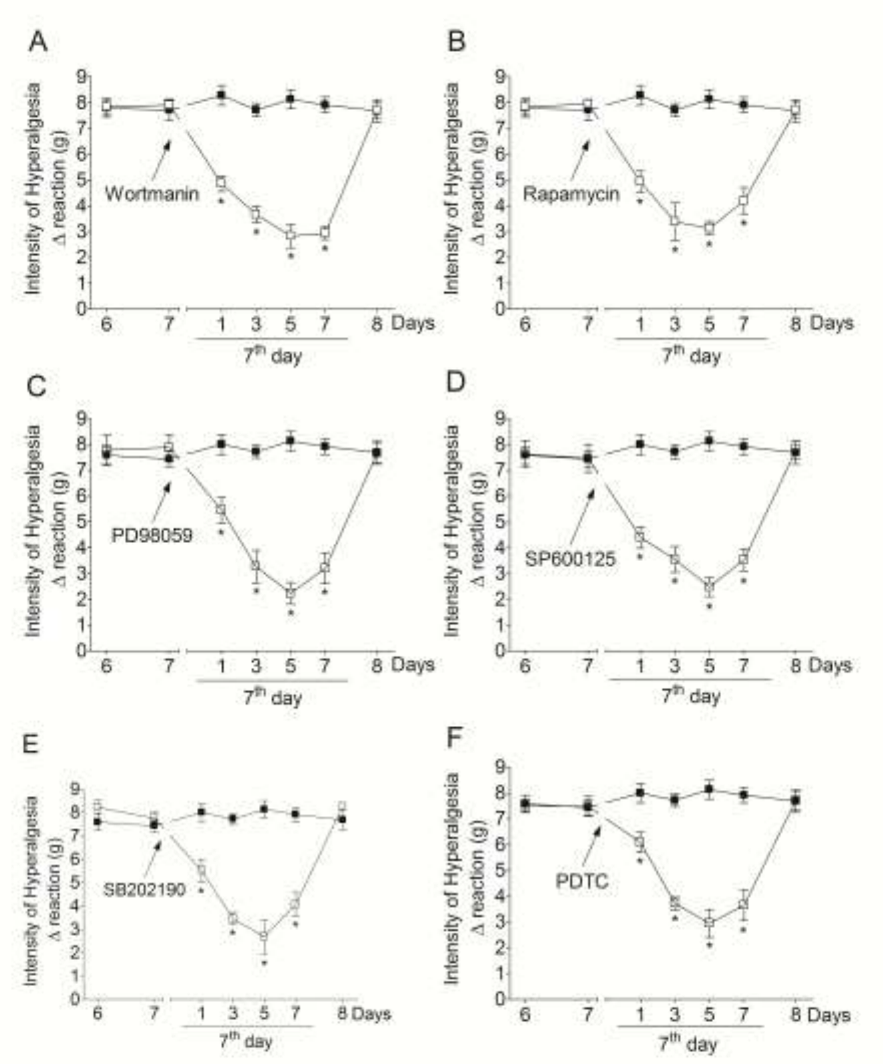

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Figure 7

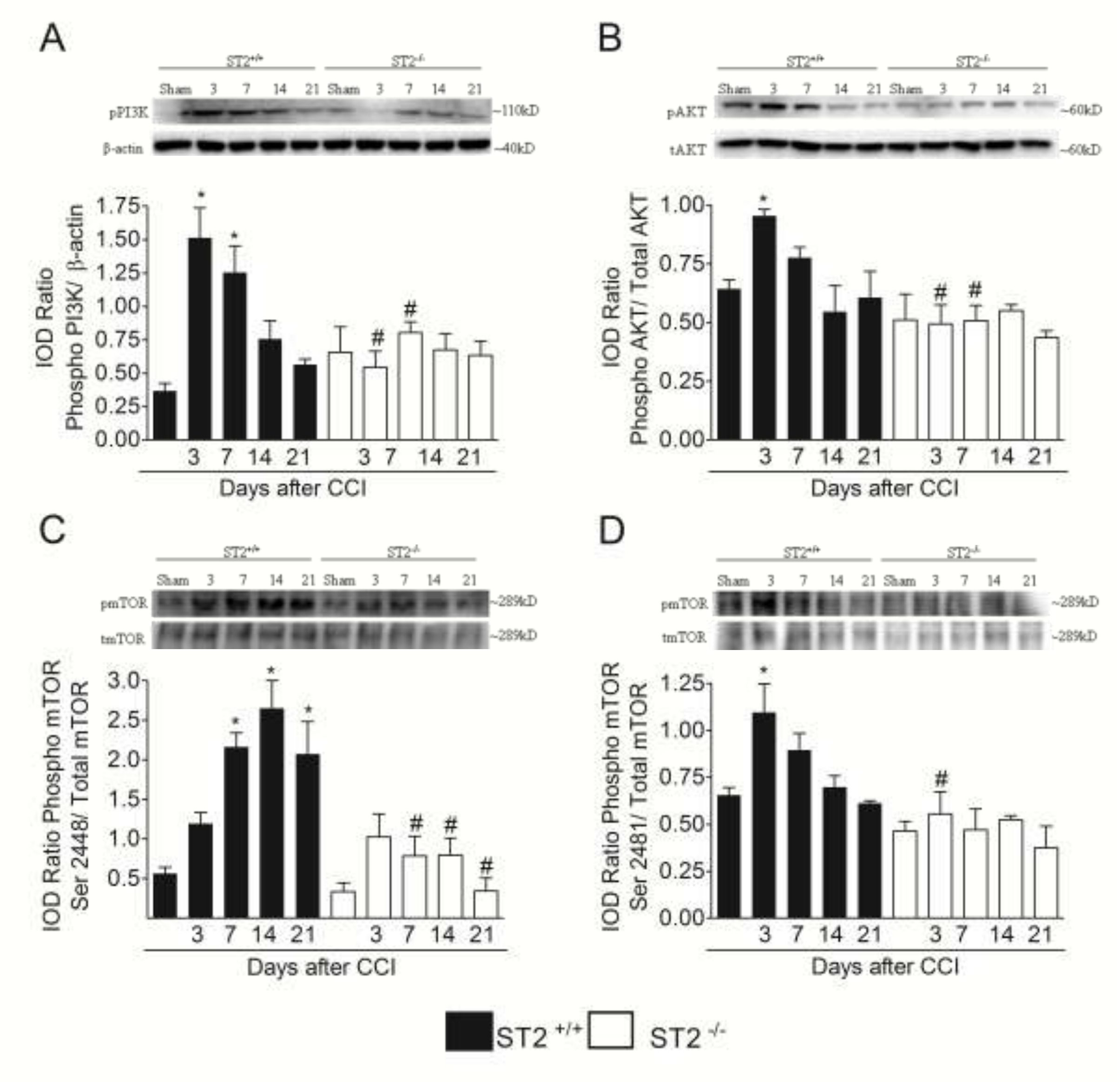

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Figure 8

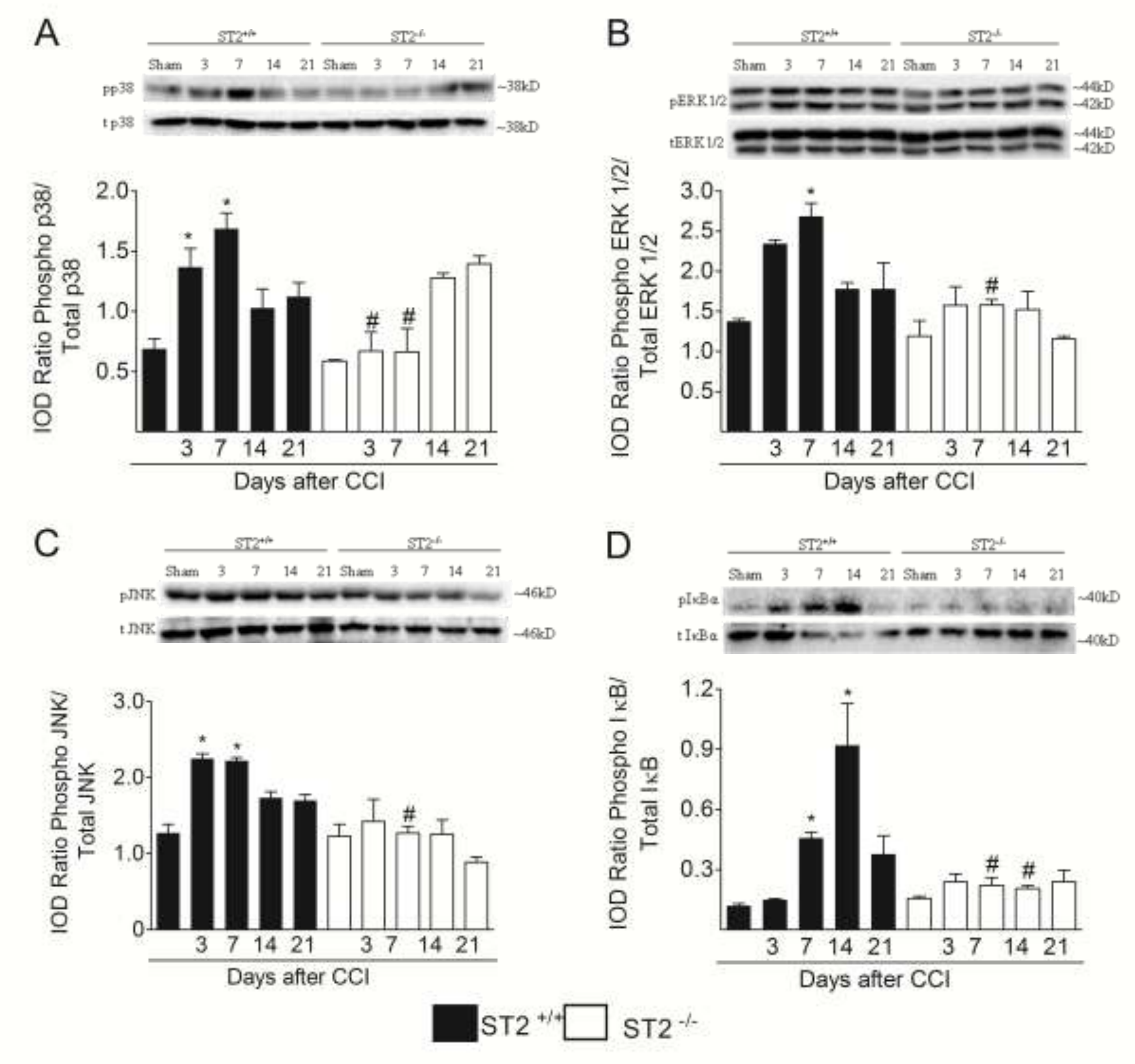


Fig. 8. Spinal activation of MAP kinases and NFκB during CCI. CCI or sham operation was induced in ST2$^{+/+}$ and ST2$^{-/-}$ mice and representative Western blot was realized 3, 7, 14 and 21 days after surgery. Representative Western blots of phospho p38, total p38 and quantification of relative levels of p38 (Panel A), phospho ERK 1/2 and total ERK 1/2 (Panel B), phospho JNK and total JNK (Panel C), Phospho IκBα (ser32) and total IκBα (Panel D). n = 4, representative of 2 separated experiments. **P* < 0.05 compared to sham group and #*P* < 0.05 compared to CCI ST2$^{+/+}$ group.

Figure 9

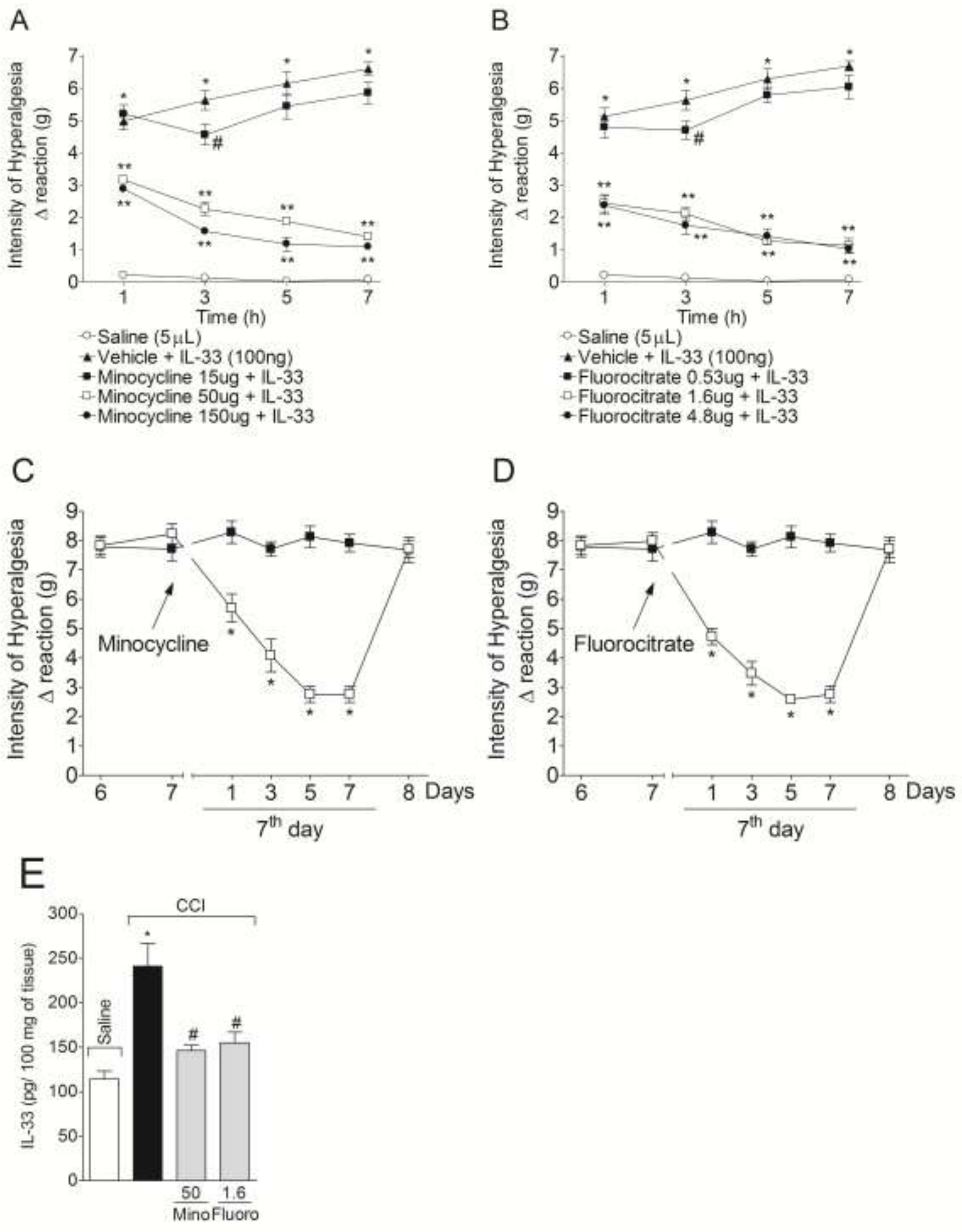


Fig. 9: The i.t. injection of IL-33 and CCI-induces mechanical hyperalgesia dependent of microglia and astrocytes in mice. Mice received co-injection (i.t.) of IL-33 (100 ng) and minocycline (microglia inhibitor; 15, 50 and 150 µg) (Panel A) or fluorocitrate (astrocytes

inhibitor; 0.53, 1.6 and 4.8) (Panel B). Mechanical hyperalgesia was evaluated at indicated time points after IL-33 injection. The mechanical hyperalgesia of CCI mice was evaluated at the 6th to 8th days after surgery. In the 7th day, mice were treated with minocycline (Panel C) and fluorocitrate (Panel D), and the spinal cord was collected for IL-33 level analysis (Panel E). n = 6, representative of 2 separated experiments.*P<0.05 compared saline group. #P<0.05 compared to vehicle + IL-33 group. **P<0.05 compared to the lower dose of drug tested. One-way ANOVA followed by Tukey's t test.

Figure 10

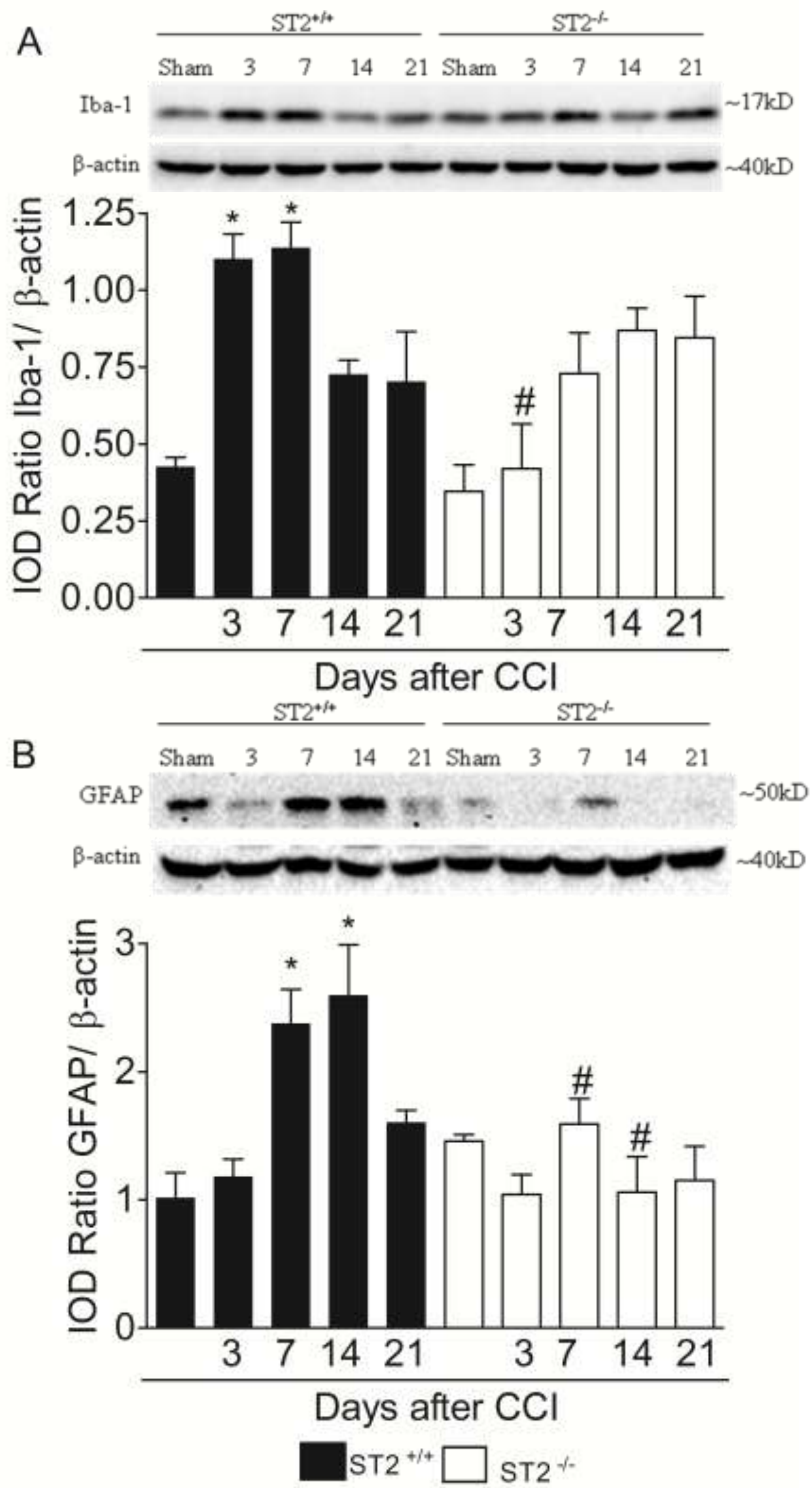
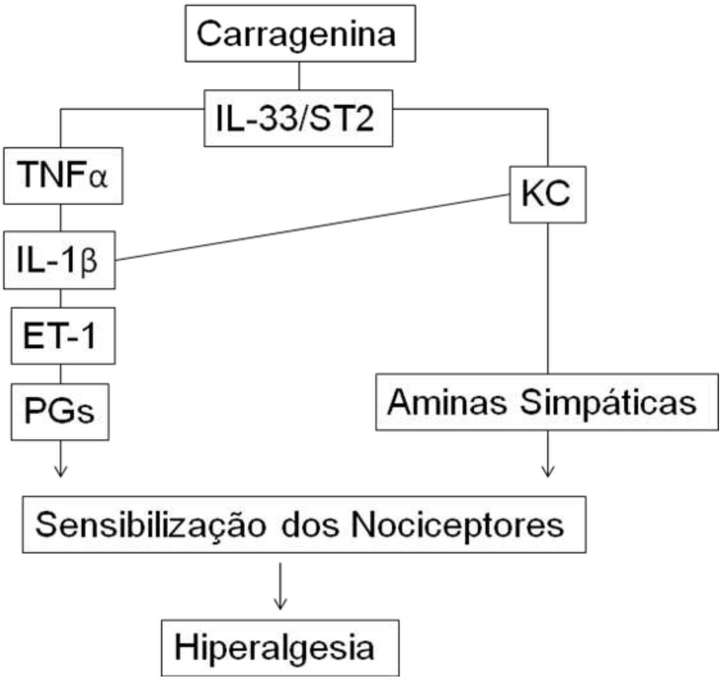


Fig. 10. Microglia and astrocytes are important cells during CCI. CCI or sham operation

was induced in ST2$^{+/+}$ and ST2$^{-/-}$ mice and representative Western blot was realized 3, 7, 14 and 21 days after surgery. Representative Western blots of Iba-1 and β-actin (Panel A) and GFAP and β-actin (Panel B). Quantification of relative levels of Iba-1 (Panel A), GFAP (Panel B). n = 4, representative of 2 separated experiments. *$P < 0.05$ compared to sham group and #$P < 0.05$ compared to CCI ST2$^{+/+}$ group.

5. Conclusões

No **primeiro artigo**, “IL-33/ST2 signalling contributes to carrageenin-induced innate inflammation and inflammatory pain: role of cytokines, endothelin-1 and prostaglandin E2” demonstramos que a IL-33/ST2 está envolvida na hiperalgesia mecânica, recutamento de leucócitos e edema induzida pela carragenina. Os mecanismos envolvem a produção de citocinas pró-inflamatórias, endotelina-1 e prostaglandina E_2. Esses dados reforçam a importância da sinalização da IL-33/ ST2 na dor e inflamação inata induzida pela carragenina.



Esquema representativo da cascata de citocinas envolvidas na hiperalgesia inflamatória. A carragenina induz a liberação sequencial de mediadores intermediários (citocinas), culminando com a produção final de prostanóides (PGs) e aminas simpáticas, as quais são capazes de sensibilizar diretamente o nociceptor. A IL-33 pode estar sendo produzida em resposta ao dano pela carragenina, além disso, estar induzindo a produção de citocinas pró-inflamatórias e endotelinas. Outro fator importante, é que a IL-33 pode estar atuando sinergicamente com a carragenina. Modificado de CUNHA et al., 2005, PNAS.

No **segundo artigo,** “Interleukin-33/ST2 signaling mediates chronic constriction injury-induced neuropathic pain in mice: Participation of spinal PI_3K, mTOR, MAP kinases, NFκB, and glia”, é possível concluir que a participação da IL-33 é necessária na dor neuropática após a injúria periférica do nervo. Após a injúria periférica ocorre a liberação de mediadores, tais como as citocinas IL-1β, TNFα e IL-33. A sinalização induzida pela IL-33/ST2 depende da ativação de PI_3K, AKT, MAP quinases (ERK, JNK e p38), NFκB e mTOR. Dessa forma, a IL-33 é um importante alvo na redução da dor neuropática, entretanto, ainda são necessários mais entendimentos para conclusões efetivas da ativação celular, bem como o papel de cada célula no processo. Com os dados de Zhao et al., 2013 e Salmond et al., 2012, podemos sugerir um possível mecanismo de interação molecular e celular e participação da IL-33, que incluem IL-33→ ST2 → PI3K ou mTORC1. PI3K → mTORC1 e C2 → citocinas pró-inflamatórias, entretanto ainda são necessário mais experimentos para determinação de uma ativação temporal no modelo de dor neuropática.